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D. J. LAWFORD: An abnormal serum component in experimental poisoning by cadmium and other metals, **7**, 109-122, Fig. 11.

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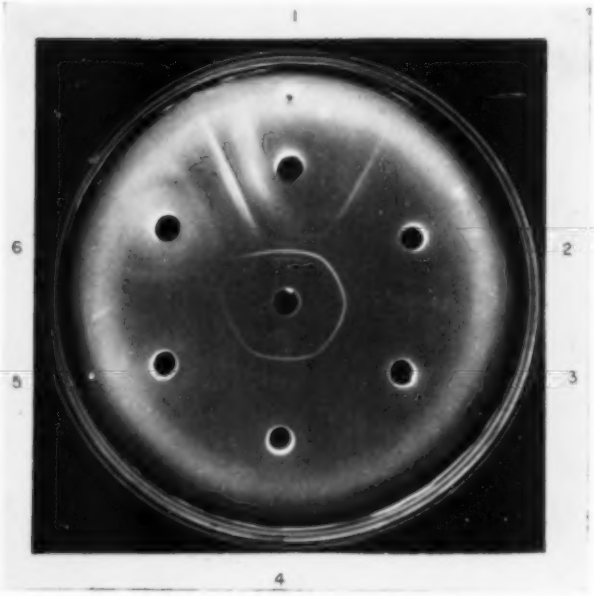

FIG. 11. Öuchterlony plate: precipitation of impurities by the addition of normal rat serum (after 14 days). Outside wells: (1) rabbit antiserum; (2) rabbit antiserum (1 vol.) + normal rat serum (0·05 vol.); (3) rabbit antiserum (1 vol.) + normal rat serum (0·5 vol.); (4) rabbit antiserum (1 vol.) + normal rat serum (1 vol.); (5) rabbit antiserum (1 vol.) + normal rat serum (3 vols.); (6) normal rat serum. Inner well: abnormal serum component (3 mg/ml in saline).

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EFFECT OF VINCAMIN ON THE NORADRENALINE CONTENT OF RAT TISSUE

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(*Received by Editors* 15 *May* 1961; *Received by Publishers* 29 *May* 1961)

Abstract—The alkaloid Vincamin considerably reduces the noradrenalin content of the brain, intestines and suprarenal glands of rats. This reduction is lasting, and can be counteracted by iproniazid pre-treatment.

VINCAMIN (Devincan® Richter) is a crystalline alkaloid isolated from *Vinca minor* L. Its chemical structure is as yet unknown; it is probably a heterocyclic compound containing an indole ring.

The pharmacological effects of the alkaloid have thus far not been completely clarified. Szporny and Szász[1] have established a two-phase blood pressure reduction on cats and rabbits as an effect of Vincamin. Immediately after intravenous administration, the blood pressure drops for a few minutes, is then re-established, and subsequently begins to drop slowly, reaching 60 per cent of its initial value in 2 hr. The same authors have also demonstrated the absence of any ganglion-blocking or adrenolytic effect.

Sedation similar to the effect of reserpine can be induced on mice and rats by the administration of considerably larger dosages of Vincamin. According to investigations carried out by Nagy and Szabó,[2] this compound has no influence on the cardiac output. Its effect on blood pressure is a result of the decrease in peripheral resistance.

Extensive clinical studies have shown that this compound is a hypotensive drug which the patients tolerate easily.

Contrary to reserpine, however, it cannot be used for the treatment of psychoses.

On the basis of the above pharmacological and clinical data, it seemed advisable to investigate the effect of Vincamin on the noradrenaline content of tissues in order to clarify its exact mechanism.

METHODS

The Vincamin used in our experiments had been prepared in the Research Laboratories of our factory and its grade of purity was checked by the paper chromatographic method of Szász *et al.*[3]

The Vincamin was dissolved in 10% ascorbic acid.

Noradrenaline isolation was carried out according to Euler.[4] Noradrenaline was isolated from tissue homogenate by hydrochloric acid–ethanol extraction. The crude extract was separated from the other substances which may affect the biological assay by phenol–hydrochloric acid chromatographic method.[5]

The noradrenaline content of the isolated material was measured according to Crawford and Outschoorn[5] by its hypertensive effect on rats. The noradrenaline sensitivity of rats anaesthetized with urethane was raised with a 1 mg/kg dose of Mecamylamine administered intravenously. In our observation Mecamylamine is better suited than the generally used Hexamethon due to the more prolonged effect of the first. The blood pressure of the rats was registered by mercury manometer with transmission.

With cerebral tissue, the determination according to the above method was carried out on the whole brain but the cerebellum. Two rats were used for each determination. The duodenum and the upper jejunum were used from the intestines (1 g). With suprarenal gland tissue, the amine concentration raising the total blood pressure of the unilateral suprarenal gland was determined.

RESULTS

In the first part of our investigation, we examined in a number of experiments the effect of 50 mg/kg Vincamin, administered intraperitoneally, on the noradrenaline content of the brain. Results obtained 2 hr after the administration of the injection are shown in Table 1. The blood pressure curves in Fig. 1 obtained from the brain extract of animals treated with Vincamin and of controls demonstrate the nature of our investigations.

TABLE 1. EFFECT OF VINCAMIN (50 MG/KG INTRAPERITONEALLY) ON THE NORADRENALINE CONTENT OF THE BRAIN*

Drug	Time (hr)	Noradrenaline	Mean
Control	–	218, 207, 227, 234, 189, 214, 190	211
Vincamin	1	70, 56, 64, 63	63
Vincamin	2	0, 0, 0, 17, 0	0(3)

* The values represent mμg/g wet tissue. 0 denotation means that the concentration has dropped below the lower value that could be measured.

It may be seen from Fig. 1 and Table 1 that the above quantity of Vincamin reduces the noradrenaline level of the brain below the measurable value (5 mμg/g). The control noradrenaline values of our experiments are in good agreement with the data published in the literature.

After having demonstrated the reducing effect of Vincamin on the cerebral noradrenaline level, we proceeded to examine this reduction as a function of time. As shown by Fig. 2, the noradrenaline level decreases considerably in the first hour, reaches a minimum value after 2 hr, and then begins to rise slowly reaching the level of the control after 48 hr. As time progresses, the noradrenaline level rises above that of the controls and reaches the level of the latter again only after 192 hr.

Table 2 shows the effect of 50 mg/kg Vincamin, administered intraperitoneally, on the noradrenaline level of the intestines and on the total amine content of the suprarenal glands. In the intestines, the noradrenaline level drops below the measurable value

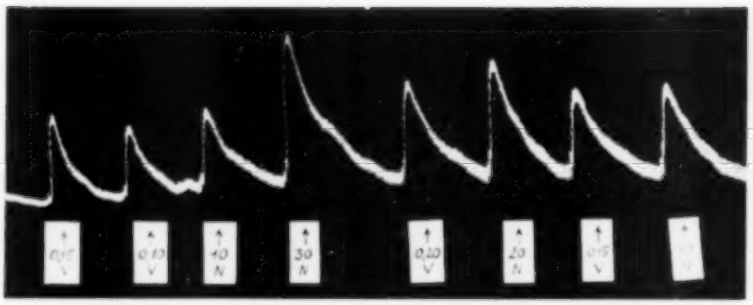

FIG. 1. (a)

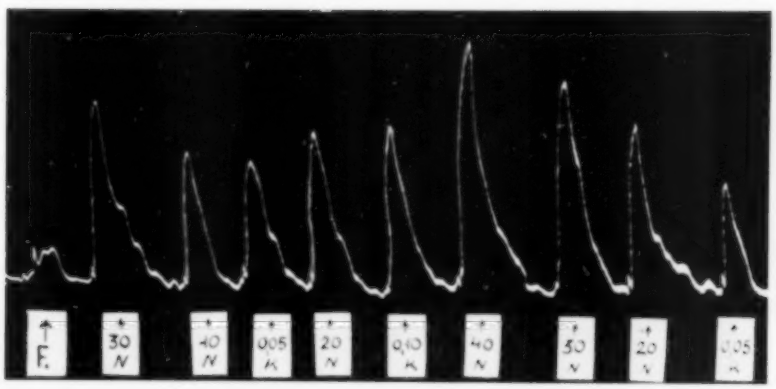

FIG. 1. (b)

FIG. 1. (a) The noradrenaline content of the brain extract of animals pretreated with Vincamin. N = noradrenaline standard/mμg. The cerebral extract of animals pretreated with Vincamin (50 mg/kg, intraperitoneally) ml/0·5 ml extract = 200 mg cerebral tissue. It can be seen that 0·15 ml V = 10 mμg noradrenaline, V = 17 mμg/g.(b) Noradrenaline content of the cerebral extract of control animals. N = Noradrenaline standard/mμg; K = cerebral extract of control animal/ml; F = physiological NaCl solution. 1 ml extract = 1050 mg cerebral tissue. It can be seen that 0·1 ml K = 20 mμg noradrenaline = 190 mμg/g.

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two hours after the administration of the injection. After 3 days' treatment of a daily dose of 50 mg/kg intraperitoneally administered Vincamin, we observed a 80 per cent reduction of the total catecholamine level on the fourth day.

The effect of Vincamin on cerebral noradrenaline level was examined 16 hr after an iproniazid (50 mg base/kg subcutaneously) pretreatment. The results obtained are

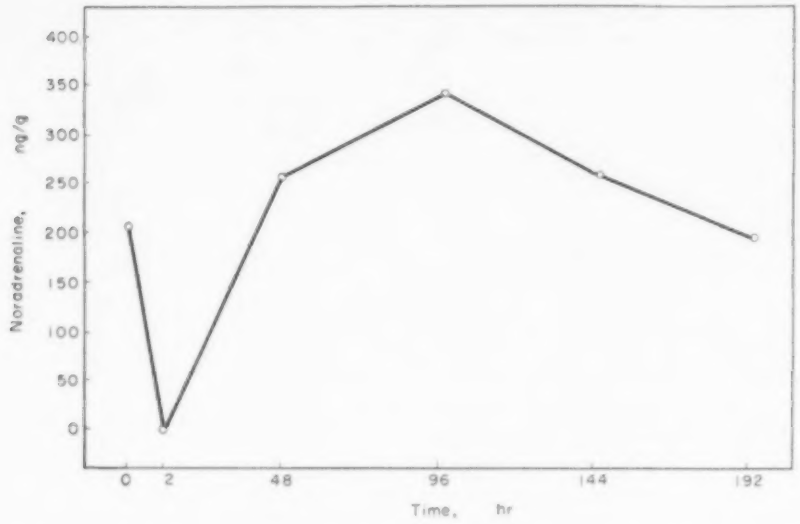

FIG. 2. Changes in the noradrenaline content of the brain as a function of the time elapsed after the intraperitoneal administration of 50 mg/kg Vincamin.

TABLE 2. THE EFFECT OF VINCAMIN ON THE NORADRENALINE CONTENT OF THE INTESTINES AND SUPRARENAL GLANDS*

Drug	Noradrenaline	Mean
	Intestine	
Control	210, 222, 254, 205	223
Vincamin	0, 0, 10, 0	0(2)
	Adrenal	
Control	56, 84, 65, 103, 80	78
Vincamin	25, 14, 12, 18	17

* Values for intestines in mμg/g tissue, for suprarenal glands in μg/unilateral suprarenal gland.

shown in Table 3. It may be seen that the iproniazid pretreatment resulted in an increase of the control values. Iproniazid pretreatment inhibits the reducing effect of Vincamin on the noradrenaline level.

DISCUSSION

On the basis of our results, we may state that Vincamin, when administered in doses that are ten times larger than those of reserpine, has a similar effect to the latter in causing a complete depletion of the cerebral and intestinal adrenaline content. For

similar effects in the suprarenal glands, a more prolonged dosage is required. Though we unquestionably observed a secondary rise of the cerebral adrenaline level in the course of our experiments, we are unable at present to explain this phenomenon.

Results obtained with iproniazid pretreatment, which acts as a monoamine oxidase inhibitor, show that Vincamin protects the cerebral noradrenaline against the action of the degrading enzyme. As Vincamin causes no further increase in the noradrenaline level as compared to the value after iproniazid pretreatment, Vincamin resembles reserpine in this respect, too.

TABLE 3. THE EFFECT OF 50 MG/KG VINCAMIN ADMINISTERED 16 HR AFTER A 100 MG/KG IPRONIAZID PRETREATMENT*

Drug	Noradrenaline	Mean
Control	192, 234, 189, 190, 204	202
Iproniazid	285, 210, 382, 245, 290	282
Iproniazid + Vincamin	197, 242, 270, 390, 253, 224	263

* Values in mμg/g wet tissue.

Summing up the results of our experiments, we may state that the effect of Vincamin on noradrenaline metabolism resembles that of reserpine. As the pharmacological and clinical effects of the two substances are only partly in agreement, these results may only partly explain the mechanism of the Vincamin effect. Clarification of the exact mechanism of the Vincamin effect and the essential investigation of the characteristics by which it differs from reserpine form the subject of further study.

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INVESTIGATIONS INTO THE CORRELATIONS BETWEEN MONOAMINE OXIDASE INHIBITION AND OTHER EFFECTS DUE TO METHYLPHENYDATE AND ITS STEREOISOMERS

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Abstract—The inhibitory activity of methylphenydate and of threo- and erythro-methylphenydate in homogenates from liver, brain and kidney were investigated. This inhibition was also demonstrated *in vivo*. The monoamine oxidase inhibition produced is of similar strength in these two cases.

The acute hypertensive actions and toxicities of the threo and erythro isomers are of equal strength; but, in agreement with other work, only the threo derivatives display a locomotor stimulant action. No parallelism could be traced between the locomotor stimulant effect of stereoisomers and their inhibition of monoamine oxidase.

MONOAMINE oxidase is known to play an important role in the functions of the central nervous system. In recent years numerous papers have reported drugs that inhibit the function of this enzyme.[1–3] Some of them, e.g. iproniazid and its derivatives, as well as compounds that are not hydrazine derivatives,[4–6] have proved useful clinically, for the treatment of states of depression.

It is also known that amphetamine, which has proved itself an efficient stimulant for the central nervous system, is a potent monoamine oxidase inhibitor: this property is thought to be connected with its therapeutic value.

Methylphenydate has been found to be an effective stimulant of the central nervous system: it is weaker than amphetamine, but its use does not lead to addiction. This drug antagonizes the sedative action of reserpine without affecting its hypotensive effect.

This paper reports investigations on the action of methylphenydate on manoamine oxidase, with special reference to its stimulant effect on the central nervous system.

MATERIALS AND METHODS

We employed methylphenydate (Centedrin®, Richter Works) containing 80 per cent of erythro and 20 per cent of threo isomers. Studies have also been undertaken with the pure stereoisomers isolated by Weisz and Dudas.[7]

Monoamine oxidase inhibition was measured *in vitro* by the colorimetric method of Udenfriend[8] using serotonin (creatinine sulphate) substrate, as well as by manometry, again using serotonin as substrate.

Manometric determinations of monoamine oxidase activity in brain tissue were carried out *in vivo* in experiments on pretreated animals.

As sources of enzymes, we used homogenized rat liver and kidney (200 mg/ml), and brain (333 mg/ml).

Locomotor stimulant action was studied in albino mice for 30 min following intraperitoneal administration of the drug: a photoelectric apparatus was used for the measurement of motility.

Blood pressure was investigated in cats under intravenous urethane anaesthesis. Toxicity was studied by the method of Litchfield-Wilcoxon, after intravenous and intraperitoneal administration in albino mice.

RESULTS

The summarized results show that methylphenydate exerts marked inhibition on monoamine oxidase (Table 1). In high concentrations ($2{\cdot}8 \times 10^{-2}$ M) the drug

TABLE 1. INHIBITION OF MONOAMINE OXIDASE ACTIVITY IN RAT LIVER*

Drug	Final conc. (M)	Inhibition, measured by colorimeter (%)	Inhibition, measured by O_2 uptake (%)
Methylphenydate	$2{\cdot}8 \times 10^{-2}$	98	100
Methyphenydate	1×10^{-2}	47	44
Threo-methylphenydate		46	41
Erythro-methylphenydate		40	45
Methylphenydate	1×10^{-3}	26	30
Threo-methylphenydate		24	32
Erythro-methylphenydate		28	25
Methylphenydate	1×10^{-4}	13	17
Threo-methylphenydate		10	18
Erythro-methylphenydate		15	18
Amphetamine	1×10^{-2}	68	73
	1×10^{-3}	36	30
Iproniazid	5×10^{-3}	98	96
	1×10^{-3}	58	64

* Values are means of four experiments. Inhibition was measured after 30 min. The substrate was 0·01 M serotonin in both cases.

produced nearly 100 per cent inhibition of enzyme function. A concentration of 1×10^{-3} M was found to give almost 30 per cent inhibition. To provide a basis for comparison, the degree of inhibition at similar concentrations of amphetamine and iproniazid have also been included. The ratio of the monoamine oxidase inhibiting action of methylphenydate to that of amphetamine equals the ratio of their locomotor stimulant effects. The table also shows that the pure threo and erythro isomers are equally potent. The values obtained by the two methods are in agreement, and they exclude any possibility that the tested materials form enzyme substrates. The time required for inhibition is the same for the three compounds (see Fig. 1).

Since it is well known that inhibition of monoamine oxidase by various compounds may be different in different organs, the three isomers have also been studied in homogenized brain and kidney, as well as in homogenized liver. The values obtained

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are shown in Fig. 2. The isomers gave the same inhibition of O_2 consumption, and their actions on the liver, brain and kidney were almost the same.

To determine the physiological significance of these experiments, we undertook *in vivo* experiments on rats. After pretreatment of the animals with intraperitoneal doses (50 mg/kg), cerebral monoamine oxidase activity was determined at intervals of $\frac{1}{2}$, 2, 6 and 16 hr later. The results obtained are presented in Fig. 3.

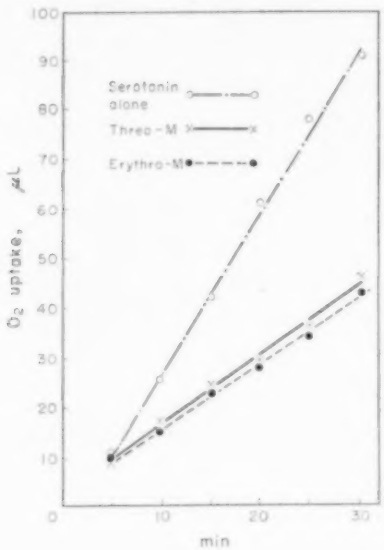

FIG. 1. Inhibition of monoamine oxidase in rat liver by stereoisomers of methylphenydate.

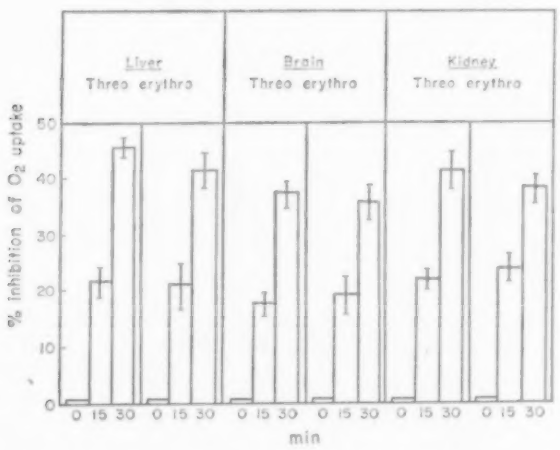

FIG. 2. Effect of methylphenydate stereoisomers on monoamine oxidase activity in rat brain, liver, and kidney homogenates. Vertical lines denote the standard errors of the assays.

As shown in the figure, monoamine oxidase is strongly inhibited by methylphenydate *in vivo* as well. The inhibitive effects of threo and erythro isomers, and the course and duration of the inhibition do not display any significant difference.

As the isomers exhibited an almost similar effect of monoamine oxidase under all experimental conditions, it appeared desirable to undertake comparative analysis of the same substances for locomotor stimulation.

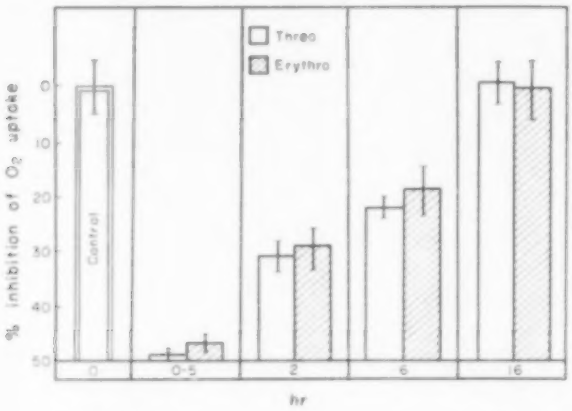

FIG. 3. *In vivo* investigation of the monoamine oxidase inhibitive activity of methylphenydate. The activity in the brain was measured at various times after the intraperitoneal injection of the drug in doses of 50 mg/kg.

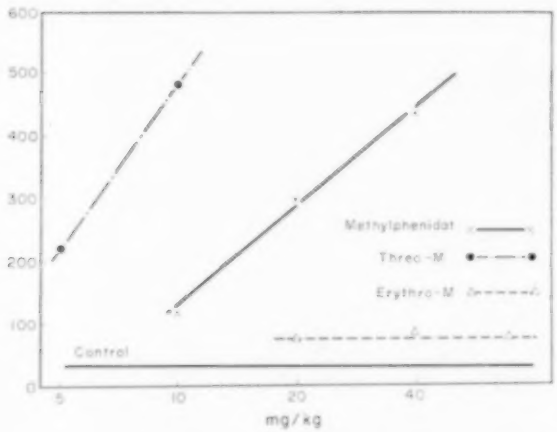

FIG. 4. Locomotor stimulating effect of methylphenydate and its stereoisomers on mice. The drugs were injected subcutaneously. Abscissa: doses (mg/kg); ordinate: number of disconnexions of light in 30 min, representing the rate of motility. Each point represents the mean value of at least ten mice.

Previous studies of this problem have shown that the threo isomer is the only effective locomotor stimulant.[9, 10] Confirmation of these results by a different method seemed to be justified. The results of our investigations are presented in Fig. 4, and the threo isomer is shown by these to be the only effective stimulant, while the erythro

isomer is ineffective. The mixture Centedrin owes its efficiency to the threo isomer which makes up 20 per cent of its contents.

Later we studied the immediate hypertensive action of the above compounds, and the results, presented in Fig. 5, show that there is no difference between the isomers.

Finally the three isomers were tested for acute toxicity: the values obtained are presented in Table 2. The LD_{50} values were essentially the same, whether administered intraperitoneally or intravenously.

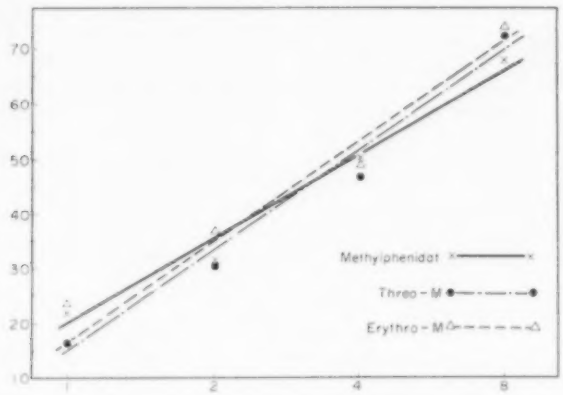

FIG. 5. Hypertensive activity of methylphenydate on cats in urethane narcosis. The ordinate represents the elevation of blood pressure as a percentage of its value before drug administration. The abscissa shows doses of the drugs (mg/kg) administered intravenously. Each point represents the mean for four animals treated with one single dose.

TABLE 2. TOXICITY VALUES OF METHYLPHENYDATE AND ITS STEREOISOMERS

Drug	LD_{50} (mg/kg injected intravenously)
Methylphenydate	41
Threo-methylphenydate	39
Erythro-methylphenydate	47

CONCLUSIONS

Methylphenydate exerts a marked inhibitive effect on monoamine oxidase under *in vivo* and *in vitro* conditions alike, as do other compounds having an anti-depressive effect. Only the threo isomer displays a locomotor stimulant effect, though all three isomers have an equal inhibiting effect on the enzyme. Only acute hypertensive action and acute toxicity of the isomers showed any parallelism with monoamine oxidase inhibition. Hence locomotor stimulant action is specific to stereo-structure, whereas the monoamine oxidase inhibition is not. Our experiments may be compared to the studies of Grana and Lilla,[11] who found that, when judged by locomotor stimulant action, *l*-amphetamine alone was active, while when tested for monoamine oxidase inhibition, the *l*- and *d*-isomers did not materially differ.

We therefore suppose that the locomotor stimulant action of these compounds is produced by a mechanism other than that responsible for the inhibition of monoamine oxidase.

Whether the anti-depressive action noted in connexion with methylphenydate and amphetamine therapy is due to the locomotor stimulant effect observed in animals or to the mechanism represented by the inhibition of monoamine oxidase, remains to be decided.

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PROTECTION OF CELLS IN TISSUE CULTURE BY MEANS OF CYSTEAMINE AND CYSTAMINE AGAINST THE ACTION OF NITROGEN MUSTARD AND X-RAYS

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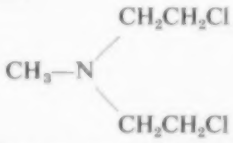
Abstract—The purpose of the present study was to decide whether cysteamine and cystamine protect cells in tissue culture against the action of nitrogen mustard and X-rays. Earle's L-strain of mouse fibroblasts was used in the experiments. Both cysteamine and cystamine afford good protection against nitrogen mustard (HN2), whereas under the same experimental conditions no definite protection of the cultures is obtained against X-irradiation with doses of 1160, 870, 580, 290 and 145 r. The results suggest that the mechanisms of the protective action of cysteamine against X-rays and against nitrogen mustards are different. It is emphasised that radio-protection of isolated cells may be obtained under other experimental conditions and with other cell types. The protection obtained against nitrogen mustard supports the view that destruction of SH groups in the cell plays a central part in the biological effect of HN2.

FOR a study of the mechanism of the protective action of cysteamine (beta-mercapto-ethylamine, $NH_2CH_2CH_2SH$) and cystamine ($NH_2CH_2CH_2S—SCH_2CH_2NH_2$) against nitrogen mustard and X-rays in animal experiments it is of great importance to decide if the protection occurs at a cellular level. The ability of cysteamine to protect isolated cells against X-irradiation has been studied by several investigators[1], but it cannot be excluded with certainty that the protection obtained may be due to anoxia produced by auto-oxidation of the cysteamine in the nutrient medium.[1, 2] Previous studies on the radio-protective effect of cystamine on tissue cultures have given negative results.[3, 4, 5]

Protection by cysteamine against the effect of nitrogen mustard has been demonstrated for plant cells *in vitro*,[6, 7] but the experimental technique employed did not exclude the possibility that it was due to a simple inactivation of the nitrogen mustard by linkage to cysteamine in the nutrient medium, i.e. outside the cells. Cystamine did not afford any protection in these experiments.

The purpose of the present investigation was therefore:

1. To study if cysteamine protects animal cells in tissue culture against the action of the nitrogen mustard HN2, i.e.



* This study was performed in the Institute of General Pathology, University of Aarhus, Denmark. It was aided by grants from the Research Council of the Danish Civil Defence Board, the Anders Hasselbalck Anti-Leukaemia Foundation and Carl Schepler and Wife's Bequest, the Irma Foundation.

under conditions where a reaction between the two substances in the nutrient medium could be excluded.

2. To study if cysteamine protects animal cells in tissue culture against the action of X-rays under experimental conditions in which anoxia of the cells caused by auto-oxidation of the cysteamine added could be excluded.

MATERIALS AND METHODS

The cells used were Earle's L-strain of mouse fibroblasts, which were grown in a slightly modified Eagle medium[8] to which 10% horse serum was added. The composition of the medium (EHS) was as follows:

Amino acids	g/l.	*Amides*	g/l.
L-arginine	0·021	L-glutamine	0·3
L-cystine	0·012		
L-histidine	0·008	*Vitamins*	mg/l.
L-isoleucine	0·026	Biotin	1
L-leucine	0·026	Choline	1
L-lysine, HCl	0·026	Folic acid	1
D,L-methionine	0·016	Nicotinamide	1
D,L-phenylalanine	0·032	Ca-pantothenate	2
D,L-threonine	0·048	Pyridoxal	1
D,L-tryptophan	0·008	Thiamin	1
L-tyrosine	0·018	Riboflavin	0·1
D,L-valine	0·048	Meso-inosit	1

Salts	g/l.	*Other components*	
NaCl	6·8	Glucose	1 g/l.
KCl	0·4	$NaHCO_3$	1·68 g/l.
$NaH_2PO_4.H_2O$	0·14	Penicillin G	10^5 I.U./l.
$CaCl_2.2H_2O$	0·265	Streptomycin sulphate	100 mg/l.
$MgCl_2.6H_2O$	0·175	Phenol Red	0·01 g/l

Serum factors
Horse serum 10%

The medium without antibiotics was sterilized by filtration through a Jena G5 glass filter. Just before use, the mixture of antibiotics was added, and the medium was bubbled with 5% CO_2 in atmospheric air, which reduced its pH level to about 7·2.

The stock cultures were grown in Roux flasks each containing 60–80 ml medium; stock cultures from 4 to 6 days old, seeded with 3 million cells per flask, were used. The cells were brought into suspension by trypsinization with a 0·05% trypsin solution for 15–30 min (trypsin 1:250 "Difco" 0·5 g/l., penicillin G 100,000 I.U./l., streptomycin sulphate 0·1 g/l., NaCl 8·0g/l., KCl 0·4 g/l., glucose 1·0 g/l., Phenol Red 0·02 g/l., $NaHCO_3$ 0·35 g/l.). The cell suspension was diluted in EHS, and a number of Carrel flasks were seeded with 2 ml per flask. Unless otherwise stated, the Carrel flasks were used for the experiments after incubation at 37° for about 20 hr. During the experiments, the medium was changed every second or third day, depending on the number of cells per flask.

After treatment of the cultures (which is described below), the cell growth was followed by daily cell counts in three Carrel flasks from each experimental group. It is stated in the literature that the L-cell can be detached from the glass wall simply by shaking the culture flasks. This was quite impossible in the present experiments, and it appeared that as early as 1 hr after the seeding of the flasks the cells adhere so densely to the glass wall that they could not be brought into suspension, even by vigorous shaking of the flasks. The cells were usually brought into suspension by the addition at room temperature of 0·5 ml of 1·25% trypsin solution (12·5 mg trypsin "Difco" 1:250 per ml dissolved in Hank's saline) to each flask without removal of the medium.

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Duplicate counts of the suspension from each flask were performed on an area of 5 mm² in a Bürker-Türk haemocytometer. The figures listed in the accompanying tables represent the average number of cells per 5 mm² obtained by duplicate counts on a total of three flasks. The number of cells per ml is obtained by multiplication of the figures by 2000 and the number of cells per flask by multiplication by 5000.

The cysteamine used was supplied by "Fluka", Switzerland, in 10 g ampoules; on receipt, the cysteamine was transferred to ampoules in amounts of 100 mg under nitrogen. Cystamine was supplied as cystamine dihydrochloride by Nutritional Biochemicals Corporation, Cleveland, Ohio.

The nitrogen mustard HN2 was supplied by A/S Ferrosan under the name of "Erasol"®, dissolved in absolute alcohol in ampoules of 10 mg.

The irradiation of the cultures was performed at the Radium Centre for Jutland. Batches of 11 Carrel flasks were irradiated at a time. The flasks were placed on a circular board, so that the distance from the centre of the beam was the same for all flasks (about 10 cm). A Philips 250 kV unit (250 kV, 15 mA; filter 0·5 mm Cu; distance 60 cm) was used. The doses were measured in air by means of a Philips universal dosimeter, with the ionization chamber placed in the centre of the beam. The doses listed in the tables are those calculated at the centres of the flasks. The dose rate was approximately 70 r/min.

RESULTS

A. Toxicity of cysteamine and cystamine

In preliminary experiments it was necessary to study the toxic effect of cysteamine and cystamine on cultures of L-cells. As it was to be expected that the effect of cysteamine depends on the size of the inoculum, the toxicity was studied in cultures with different initial cell counts. Cysteamine or cystamine was dissolved in EHS immediately before the addition to cultures 20 hr old. At subsequent changes of medium, cysteamine or cystamine was added in the same concentration as at the start of the experiment. The results of a series of experiments are shown in Table 1, in which, as in all subsequent tables, the figures indicate the cell counts per 5 mm² in the haemocytometer, each figure being the average of duplicate counts on three flasks (see also Materials and Methods).

In the first three experiments, the toxicity of cysteamine was studied at different initial cell counts in the flasks. It is seen that at an initial cell count of 47 (experiment I) a cysteamine concentration of 2×10^{-3} M killed all the cells (i.e. the cell count fell to 0). At an initial cell count of 17 (experiment II), all the cells were killed at a concentration of $1{\cdot}0 \times 10^{-3}$ M, and when the initial cell count was reduced to 5 (experiment III), all cells were killed at a concentration of $0{\cdot}5 \times 10^{-3}$ M. A repetition of the experiments showed the same toxicity and the same relationship between the initial cell count and the toxicity.

In experiment IV, the toxicity of cystamine was studied in cultures with an initial cell count of 21. It is seen that a concentration of $0{\cdot}5 \times 10^{-3}$ M (converted to the concentration of the cystamine base, since the hydrochloride was used) killed all the cells. Thus, in molar amounts, the toxicity of cystamine was about twice as high as that of cysteamine (cf. experiments II and IV). However, as one molecule of cystamine by reduction forms two molecules of cysteamine, the toxicity of the two substances, calculated on the basis of their content of sulphur, is nearly identical.

Cysteamine at a concentration higher than that stated in Table 1 could be used in the radiation protection experiments, if it was allowed to exert its action for only a limited period. The result of one experiment is shown in Table 2. In all four groups, the cysteamine concentration was 3×10^{-3} M, but the cysteamine-containing medium was replaced by a cysteamine-free one after 15, 30, 60 and 90 min at

TABLE 1. CELL COUNTS IN CULTURES OF L-CELLS TREATED WITH CYSTEAMINE OR CYSTAMINE AT VARIOUS CONCENTRATIONS*

Experiment no.	Days	Concentration of cysteamine or cystamine					Controls
		2×10^{-3}M	10^{-3}M	$\frac{1}{2} \times 10^{-3}$M	$\frac{1}{4} \times 10^{-3}$M	$\frac{1}{8} \times 10^{-3}$M	
I	0			47		—	47
Cysteamine	1	14	63	86	85	—	95
	2	5	62	134	187	—	199
	3	1	91	254	362	—	363
	4	2	107	—	—	—	—
	5	—	176	—	—	—	—
	6	0	244	—	—	—	—
II	0			17			17
Cysteamine	1	—	16	28	34	29	31
	2	—	7	32	63	74	83
	3	—	1	46	122	164	173
	4	—	0	82	247	292	290
	5	—	1	116	409	538	519
	6	—	0	153	559	—	—
III	0			5			5
Cysteamine	1	—	4	8	9	9	9
	2	—	1	6	12	18	30
	3	—	1	6	18	39	55
	4	—	0	4	23	76	111
	5	—	0	1	52	152	187
	6	—	—	0	80	283	378
	7	—	—	0	123	444	—
IV	0			21			21
Cystamine	1	0	0	0	24	35	42
	2	0	0	0	24	64	89
	3	0	0	0	13	88	165
	4	—	—	—	—	—	—
	5	—	—	0	14	471	560

* The substances were added to the cultures on day 0, and at the change of medium the substances were added in the same concentration as at the start of the experiment. Experiments I–III show the effect of cysteamine on cultures with various cell counts. Experiment IV shows the effect of cystamine at an initial cell count of 21. The figures listed in this and the following tables represent the average number of cells obtained by duplicate counts on three flasks from each group (see also Materials and Methods).

TABLE 2. EFFECT OF CYSTEAMINE ON THE CELL COUNT*

Days	Duration of treatment in min				Controls
	15	30	60	90	
0			26		26
1	57	54	49	38	58
2	145	143	103	77	151
3	276	274	260	162	280

* Cysteamine was added to the cultures in a concentration of 3×10^{-3}M on day 0, when the cysteamine-containing medium was replaced by cysteamine-free medium at 15, 30, 60 and 90 min after the addition.

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37 °C. It is seen that no demonstrable inhibition of growth was obtained when the change was made after 15 or 30 min. A slight inhibition of growth was observed when the change was made after 60 min, while change after 90 min resulted in a considerable inhibition. Since in the subsequent protection experiments the time of cysteamine exposure was chosen as 60 min, it seemed possible to use cysteamine concentrations of up to 3×10^{-3} M.

B. Protection with cysteamine and cystamine against the effect of HN2

In order to study this problem it was necessary to use experimental conditions which excluded the possibility of inactivation of HN2 by linkage to cysteamine or cystamine in the medium. Each Carrel flask was seeded with about 120,000 cells, i.e. a cell count of 24, and about 20 hr later the flasks were treated as follows:

Group I Cysteamine or cystamine alone
Group II Cysteamine or cystamine before HN2
Group III HN2 alone
Group IV No addition to the medium (controls).

Apart from the addition of these substances, all four groups were treated in exactly the same manner.

After empyting the flasks, 2 ml of fresh medium was added, containing cysteamine or cystamine in groups I and II, but not in groups III and IV. The flasks were then incubated at 37° for 1 hr, following which they were emptied again and placed in a rack with the mouths downwards for a few minutes to allow the remaining nutrient medium to drip off. Each flask was then rinsed with 1 ml of Hank's saline and again placed in the rack as before. Finally, 2 ml nutrient medium was added; it consisted of 10% Hank's saline and 90% EHS; in groups I and III with addition of HN2 to the desired concentration and in groups I and IV without any addition. The HN2 solution was prepared immediately before its addition to the cultures by dilution with Hank's saline to 10 times the final concentration. The final dilution (ten times) was done by mixture of 1 part of HN2 solution and 9 parts of EHS. The cultures were then incubated at 37 °C, and daily cell counts were performed on three flasks from each group.

The results of two experiments are shown in Table 3. The protective action of cysteamine was studied at a concentration of 3×10^{-3} M. It is seen that both experiments revealed protection (cf. groups II (protected) and III (unprotected)).

In the first experiment, the cysteamine concentration used showed only a slight toxic effect or none at all (cf. groups I (cysteamine-treated) and IV (untreated)), while the toxic effect observed in the second experiment was relatively great; but nevertheless protection was also obtained in the latter experiment.

To obtain a rough measure in the degree of protection, groups of protected and unprotected cultures were treated with varying concentrations of HN2, and for each concentration cell counts were performed on three protected and three unprotected cultures 7 and 14 days after treatment.

The results appear from Table 4. The protection by cysteamine in concentrations of $3{\cdot}0 \times 10^{-3}$ and $1{\cdot}5 \times 10^{-3}$ M and by cystamine in a concentration of $0{\cdot}75 \times 10^{-3}$ M was studied. A comparison of protected and unprotected cultures shows that in all three experiments the HN2 concentration required to give the same effect had to be four times as high in the protected as in the unprotected cultures.

C. Protection with cysteamine and cystamine against the effect of X-irradiation

The technique employed was that described above, with the exception that the rinsing after initial incubation of the cultures at 37 °C for 1 hr (after the addition of cysteamine or cystamine to the medium) was done with 1 ml of EHS instead of with 1 ml of Hank's saline. After the rinsing, no fluid was added until after the irradiation.

TABLE 3. CELL COUNTS IN CULTURES TREATED WITH CYSTEAMINE AND/OR NH2*

	HN2 $\frac{1}{64} \times 10^{-4}$M				HN2 $\frac{1}{8} \times 10^{-4}$M			
	I	II	III	IV	I	II	III	IV
0	26				36			
1	34	21	27	28	16	16	26	50
2	78	26	21	74	16	16	20	127
3	145	40	18	151	35	19	15	193
4	305	72	30	324	124	18	12	362
5	—	139	34	—	138	29	11	—
6	650	179	45	671	265	31	13	676
7	765	201	53	783	301	40	10	739
8	—	342	61	—	—	34	8	—
9	—	373	93	—	—	66	12	—
10	—	478	169	—	—	120	11	—
11	—	497	230	—	—	215	12	—

* Cysteamine, 3×10^{-3}M. The treatment was performed on day 0 (i.e. about 20 hr after the seeding) as follows:
I Cysteamine alone
II Cysteamine before HN2
III HN2 alone
IV Controls

TABLE 4. CELL COUNTS IN CULTURES 7 AND 14 DAYS AFTER TREATMENT WITH CYSTEAMINE OR CYSTAMINE AND/OR HN2*

Concentration of HN2	Cysteamine 3×10^{-3}M				Cysteamine $1{\cdot}5 \times 10^{-3}$M				Cystamine $0{\cdot}75 \times 10^{-3}$			
	7 days		14 days		7 days		14 days		7 days		14 days	
	+	−	+	−	+	−	+	−	+	−	+	−
$\frac{1}{4} \times 10^{-4}$M	0	0	0	0	0	0	0	0	3	0	1	0
$\frac{1}{8} \times 10^{-4}$M	3	0	34	0	4	0	2	0	11	1	10	1
$\frac{1}{16} \times 10^{-4}$M	24	0	321	0	14	1	119	0	22	3	242	2
$\frac{1}{32} \times 10^{-4}$M	126	3	609	20	107	4	494	2	143	9	657	11
$\frac{1}{32} \times 10^{-4}$M	177	10	853	162	324	10	—	27	324	18	—	199

* + signifies treatment with cysteamine or cystamine before HN2 in various concentrations.
− signifies HN2 without previous treatment with cysteamine or cystamine.

It is emphasised that, just like the HN2 treatment considered in section B, X-irradiation was performed at room temperature. As no X-ray unit is available in the Institute of General Pathology, the cultures had to be transported to another institute (see above), where the irradiation was performed. About $2\frac{1}{2}$ hr elapsed from the time the cultures were taken out of the incubator until they were placed there again after the irradiation and addition of 2 ml EHS.

All the experimental groups were treated in exactly the same manner, apart from the addition of cysteamine or cystamine and the irradiation.

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The experimental set-up was as follows:

Group I Cysteamine or cystamine alone
Group II Cysteamine or cystamine before irradiation
Group III Irradiation without any pre-treatment
Group IV Controls.

In two preliminary experiments an X-ray dose of 580 r was chosen. No protection was obtained in these experiments. As the experimental conditions were not quite the same as in section B, it was decided to investigate protection both against HN2 and against irradiation under identical experimental conditions.

The experimental set-up in these experiments (Tables 5 and 6) was as follows:

Group I Cysteamine or cystamine alone
Group II Cysteamine or cystamine before irradiation (580 r)
Group III Irradiation (580 r) alone
Group IV Controls
Group V Cysteamine or cystamine before $\frac{1}{12} \times 10^{-4}$ M HN2
Group VI $\frac{1}{12} \times 10^{-4}$ M HN2 alone

TABLE 5. CELL COUNTS IN CULTURES TREATED ON DAY 0

Days	Cystamine $1{\cdot}5 \times 10^{-3}$M; X-ray dose 580 r; HN2 $\frac{1}{12} \times 10^{-4}$M						Cystamine $0{\cdot}75 \times 10^{-3}$M; X-ray dose 580 r; HN2 $\frac{1}{12} \times 10^{-4}$M					
	I*	II	III	IV	V	VI	I*	II	III	IV	V	VI
0	31						24					
1	19	14	29	43	12	23	48	29	27	51	37	26
2	30	9	47	88	14	17	104	42	46	113	31	22
3	61	9	47	204	—	—	256	54	59	269	—	—
4	136	16	50	404	14	5	477	57	55	412	42	7
5	—	—	—	—	—	—	—	—	—	—	—	—
6	419	16	52	852	12	5	—	80	78	—	59	7
7	—	18	69	—	—	—	—	110	99	—	—	—
8	—	12	88	—	39	4	—	150	150	—	93	5
9	—	15	107	—	—	—	—	169	169	—	—	—
10	—	16	193	—	104	3	—	272	257	—	271	5
11	—	29	223	—	—	—	—	321	309	—	—	—
12	—	—	—	—	—	—	—	—	—	—	—	—
13	—	172	501	—	349	4	—	525	480	—	450	4

* I Cystamine alone
II Cystamine before irradiation (580 r)
III Irradiation (580 r) alone
IV Controls
V Cystamine before HN2, $\frac{1}{12} \times 10^{-4}$M
VI HN2, $\frac{1}{12} \times 10^{-4}$M, without pretreatment

The results of two experiments with cystamine are shown in Table 5. In the first experiment, a cystamine concentration of $1{\cdot}5 \times 10^{-3}$ M, which has a relatively great toxic effect, was used. The experiment revealed good protection against HN2 in spite of the toxic effect of the substance, while it did not seem to afford any protection against X-irradiation. In the second experiment, a cystamine concentration of $0{\cdot}75 \times 10^{-3}$ M, which had no toxic effect, was used. Once more, good protection against HN2, but none against X-irradiation, was obtained.

The results of three experiments with cysteamine are shown in Table 6. In all three experiments, a cysteamine concentration of $1{\cdot}5 \times 10^{-3}$ M was used, but to decide if protection against irradiation could be obtained if the cells were treated at different times after seeding (see Discussion), the treatment of the cells were performed at 2, 24 and 48 hr after the seeding.

TABLE 6. CELL COUNTS IN CULTURES TREATED AS IN TABLE 5 WITH MODIFICATIONS*

Cysteamine $1{\cdot}5 \times 10^{-3}$M; X-ray dose 580 r; HN2 $\frac{1}{10} \times 10^{-4}$M

Days	Treatment at 2 hr after seeding						Treatment at 24 hr after seeding						Treatment at 48 hr after seeding					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI	I	II	III	IV	V	VI
0	22						28						21					
1	20	19	15	18	16	11	41	30	32	47	40	38	31	21	23	32	—	—
2	35	25	25	35	15	9	89	47	46	111	27	20	48	29	25	56	—	—
3	67	34	42	56	15	5	177	54	53	203	27	17	93	38	27	107	—	—
4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	185	56	54	157	28	3	390	77	70	440	35	10	193	37	40	232	—	—
6	239	55	58	239	33	5	—	78	64	—	—	—	222	26	39	277	—	—
7	—	63	75	—	49	8	—	80	77	—	38	10	298	28	30	350	—	—
8	—	69	78	—	68	6	—	96	77	—	—	—	—	42	42	—	—	—
9	—	80	101	—	—	—	—	134	121	—	67	8	—	37	36	—	—	—
10	—	110	107	—	131	10	—	131	142	—	—	—	—	45	47	—	—	—
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	—	164	186	—	249	12	—	249	240	—	225	11						
13	—	—	—	—	—	—	—	291	251	—	—	—						

* Cysteamine ($1{\cdot}5 \times 10^{-3}$M) was used in place of cystamine: the other conditions are as in Table 5. As shown in this table, the treatment was performed at different times after the seeding of the cultures.

Only in the first two experiments was the protection against HN2 studied at the same time. It is seen that in both experiments good protection against HN2, but none against irradiation, was obtained. Nor was any protection against X-irradiation obtained in the third experiment. However, it should be noticed that cysteamine seemed to exert a slight inhibitory action on the growth of the cultures in the last two experiments listed in the table.

The protective action of cysteamine and cystamine against lower and higher X-ray doses were also studied.

In four experiments the X-ray dose applied was 290 r. In these experiments, cystamine concentrations of $0{\cdot}75 \times 10^{-3}$ and $0{\cdot}38 \times 10^{-3}$ M and cysteamine concentrations of $0{\cdot}5 \times 10^{-3}$ and $0{\cdot}75 \times 10^{-3}$ M were used.

One experiment was suggestive of a slight protective effect by $0{\cdot}38 \times 10^{-3}$ M cystamine, while the remaining three experiments did not reveal any protection.

For three experiments an X-ray dose of 145 r was used. Neither did these experiments reveal any evidence in favour of a protective effect. Protection against HN2 was studied in two of the experiments; both showed distinct protection. Finally, the results of two experiments, in which X-ray doses of 870 and 1160 r were used, are shown in Table 7. Again, no protection against irradiation, but good protection against HN2 was obtained.

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In recapitulation, it may be said that it was not possible to obtain significant protection against the effect of X-irradiation under the experimental conditions used, whereas good protection against HN2 could be obtained under exactly the same conditions.

TABLE 7. CELL COUNTS IN CULTURES TREATED AS IN TABLE 5*

Days	Cystamine $0{\cdot}75 \times 10^{-3}$M; X-ray dose 870 r; HN2 $\frac{1}{18} \times 10^{-4}$M						Cystamine $0{\cdot}75 \times 10^{-3}$M; X-ray dose 1160 r; HN2 $\frac{1}{18} \times 10^{-4}$M					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
0	31						30					
1	59	32	36	55	—	—	44	28	24	45	—	—
2	121	41	46	127	—	—	111	29	32	121	30	20
3	—	—	—	—	—	—	192	—	—	219	—	—
4	429	39	40	456	35	16	375	30	31	412	32	10
6	—	20	22	—	—	—	—	20	20	—	—	—
8	—	15	13	—	98	11	—	16	15	—	59	8
11	—	14	10	—	—	—	—	11	10	—	130	7
13	—	13	18	—	—	—	—	10	9	—	290	3
18	—	79	103	—	—	—	—	7	7	—	—	—
20	—	121	114	—	—	—	—	10	9	—	—	—
22	—	180	164	—	—	—	—	4	11	—	—	—

* The X-ray doses were 870 r and 1160 r; HN2, $\frac{1}{18} \times 10^{-4}$M; Cystamine, $0{\cdot}75 \times 10^{-3}$M

DISCUSSION

For a more detailed discussion of previous investigations on the toxic and protective effects of cysteamine the reader is referred to a recently published survey.[1] The toxicity, as shown above, depends on the number of cells present in the cultures. This dependence should be expected, especially if the explanation of the toxic effect is that offered by Eldjarn and Pihl,[11] viz. that cysteamine forms mixed disulphides with SH-bearing proteins in the cell, resulting, *inter alia*, in an inactivation of enzymes whose activity depends on the presence of a free SH group.

On the assumption that a certain number of SH groups per cell are to be blocked in order to obtain a toxic effect, this dependence between the toxicity and the number of cells in the cultures should be expected.

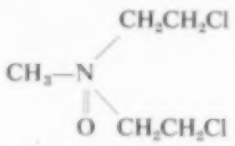
As regards the possibility of protecting the L-cell against the action of HN2 the present experiments showed that both cysteamine and cystamine afford good protection under conditions where inactivation of HN2 by linkage to cysteamine or cystamine in the medium can be excluded.

On the basis of the investigations of Eldjarn and Pihl,[11] it is reasonable to assume that cysteamine is firmly bound as mixed disulphides to SH-bearing proteins in the cells. That cysteamine is bound in the cells also appeared in my experiments from the fact that cysteamine could not be removed from the cells if they were kept in a cysteamine-free medium for $1\frac{1}{2}$ hr at room temperature. No diffusion of cysteamine from the cells into the cysteamine-free medium seems to occur.

As stated in a previous survey,[1] a possible explanation of the protective effect is therefore that SH groups on the proteins of the cell are protected against the action of HN2 through the formation of mixed disulphides with cysteamine, since HN2 will have a considerably lower affinity for S—S bonds than for the ionised free SH groups.

Accordingly, the mechanism of action should then consist in a change in the point of attack of HN2 in the cell: SH groups are protected, while the strongly reactive HN2 attacks groups which are of less significance for the continued existence of the cell.

The possibility of protecting cells *in vitro* against the effect of nitrogen mustard by means of cysteamine and cystamine has also been studied by Deysson and Truhaut.[6, 7] They studied the protective action of the two substances against the effect of HN3 on *Allium cepa* roots and against the effect of nitromine



on *Pisum sativum*. In both cases, they found good protection by cysteamine, but no protection by cystamine. However, in their experiments the nitrogen mustard was mixed *in vitro* with cysteamine or cystamine in isotonic saline (Knop solution), and these mixtures were added to the cultures. The protection obtained by cysteamine must be supposed to be due to simple inactivation of the nitrogen mustard in the medium, while the lack of protection by cystamine may be explained by the fact that this substance only to a slight extent inactivates nitrogen mustard.* Simultaneous addition of cystamine and nitrogen mustard to the cells excludes the possibility that cystamine may react with the cellular elements before the nitrogen mustard has exerted its action. Accordingly, protection by the mechanism of action outlined above cannot either be obtained by the experimental technique employed by Deysson and Truhaut.

If the mechanism of the protective action of cysteamine is that proposed above, the results of the present experiments also lend support to the view that the destruction of SH groups in the cells plays a central role in the biological effects of nitrogen mustard.

As regards the possibility of protecting L-cell cultures against X-rays, the present studies show that no definite protection could be obtained with cysteamine or cystamine, while these substances afforded good protection against HN2 under exactly the same experimental conditions. Accordingly, the experiments suggest that the mechanism of the radio-protective action by cysteamine and cystamine differs from that of their protective action against HN2. According to the theory advanced by Eldjarn and Pihl,[11, 12] the mechanism should in both cases be that the substances form mixed disulphides with protein-bound SH groups and thus protect these proteins against the effect of both X-rays and HN2.

A review of the literature[1] seems to show that previous investigations on the possibility of protecting tissue cultures against the action of X-rays by means of cystamine have all given negative results,[3, 4, 5] but protection has been obtained with isolated thymocytes.[13] Cysteamine has afforded protection of various cell types, but it cannot be definitely excluded that the protection obtained in these cases was due to anoxia in the nutrient medium produced by auto-oxidation of the cysteamine added.[2]

Anoxia of the cells caused by auto-oxidation of the cysteamine added is unlikely under the present experimental conditions, since the cells were rinsed with freshly

* When cysteamine is mixed with HN2 in Hank's saline, an appreciable inactivation of HN2 occurs within 2–3 min, whereas cystamine inactivates HN2 at a much slower rate (unpublished experiments).

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aerated medium after the treatment with cysteamine and since the irradiation was then performed without any medium covering the cells.

Nevertheless further experiments are necessary before definite conclusions are drawn as to the possibility of protecting the L-cell with cysteamine against irradiation. So far it is only possible to conclude that no protection can be obtained under the present experimental conditions,* and it must be emphasized that the L-cell used cannot be regarded as a normal animal cell, since a rough count of its chromosomes showed that it had a modal chromosome number of 62, while the normal diploid chromosome number in the mouse is 40.

* As the maximum radio-protective effect of cysteamine in experiments with mice is obtained if the compound is injected shortly (from 0 to 30 min) before the irradiation, a couple of experiments were done, in which the cultures were irradiated from 20 to 30 min after the addition of cystamine or cystamine. No protection was obtained.

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EFFECTS OF NITROGEN MUSTARD ON PROTEIN AND NUCLEIC ACID SYNTHESIS IN MOUSE FIBROBLASTS GROWING *IN VITRO*

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Abstract—Nitrogen mustard, added at 0·1 μg/ml to the culture medium of mouse fibroblasts growing *in vitro*, led to rapid cessation of cell division and of DNA synthesis. RNA and protein synthesis continued at a rapid rate for several days, leading to greatly enlarged cells. Although the accumulation of DNA showed an abrupt halt shortly after nitrogen mustard addition, the inhibited cells nevertheless had up to twice the amount of DNA found in a normal, rapidly growing cell. Cells whose growth was limited by depriving them of an essential amino acid, leucine, showed no such effect. It is suggested that nitrogen mustard halts the division process at a time after the replication of DNA that occurs in preparation for division.

INTRODUCTION

THE growth-inhibitory and cytotoxic effects of nitrogen mustard (HN2)* have been widely believed to be due to blocking of DNA synthesis, perhaps by a process of cross-linking.[1, 2] Bodenstein and Kondritzer[3] demonstrated a block of DNA synthesis in salamander embryos exposed to HN2, while RNA accumulation continued to occur in a normal manner. Goldthwait[4] obtained similar results, studying the incorporation of ^{14}C-formate or ^{15}N-adenine into RNA and DNA of rat intestine. Herriott[5] has also obtained evidence, utilizing *E. coli*, that HN2-blocked cell division and DNA synthesis without interfering with RNA synthesis. Nevertheless, some question remains[6] as to whether the effects of HN2 on mammalian systems can be ascribed solely to an effect on DNA synthesis. It also seems clear that while polyfunctional alkylating agents are more potent than monofunctional agents with respect to growth-inhibitory activity,[7] monofunctional alkylating agents are at least as potent for other effects, such as mutagenesis.[8] Since the monofunctional compounds could not be participating in cross-linking reactions, mutagenic effects, at any rate, could not be explained by a cross-linking of DNA molecules.

In an effort to obtain quantitative data on the effects of HN2 on a rapidly growing mammalian cell, the effects of the drug were studied using mouse fibroblasts (Earle's "L" cell) growing *in vitro*. Cell counts, protein, RNA and DNA synthesis were examined.

METHODS

The procedures used were similar to those described by Saltzman.[9] Replicate cell inocula were prepared in 8-oz prescription bottles, stoppered with silicone stoppers,

* Abbreviations: HN2, nitrogen mustard; DNA, deoxynucleic acid; RNA, ribonucleic acid.

with 25 ml of Eagle's medium [10] containing 10 per cent beef serum. For analysis, the supernatant medium was poured off, and the adherent cells were gently rinsed with two 10-ml portions of a balanced salt solution. Individual cell counts were obtained on three bottles in each experimental group by scraping the cells in 10 ml of filtered 0·9% saline containing 0·1% trypsin, pipetting up and down vigorously a few times to disperse the cells, and immediately counting the cells in a Coulter electronic cell counter. The saline suspension was diluted ten-fold for counting when necessary. This procedure, using a weak concentration of trypsin, was found (by microscopic examination) to give well-dispersed, single cells with no signs of cell lysis. The entire scraping and counting procedure required less than 2 min.

Assays for protein, RNA and DNA were obtained from other bottles. The rinsed bottles were chilled to 3–5 °C, and the contents of from three to twenty bottles were scraped with successive 5-ml portions of ice-cold 8% perchloric acid. The acid-insoluble material in each group was pooled and centrifuged in the cold, and the supernatant solution discarded. Lipids were removed from the cold acid-insoluble material by extracting successively with 10-ml portions of alcohol–water (4:1), alcohol–ether (3:1), and ether. The residue was then treated with 3 ml of 6% perchloric acid for 20 min at 90 °C to hydrolyse the purine components of the nucleic acids. After cooling, the tubes were centrifuged and the hot acid-soluble fraction was separated from the residue.

Protein was determined on the residue after an alkaline digestion by the procedure of Oyama and Eagle.[11] Ribose was determined in an aliquot of the hot acid-soluble supernatant solution using the orcinol reaction of Volkin and Cohn.[12] Deoxyribose was determined in an aliquot utilizing the procedure described by Burton.[13] Since deoxyribose contributes a small amount of color to the orcinol reaction (10 μmoles of deoxyribose is equivalent to 0·66 μmoles of ribose), the ribose determinations were corrected by an appropriate amount. All colorimetric determinations were performed in duplicate.

RESULTS

The effects of various concentrations of HN2 on rapidly growing fibroblasts are indicated in Fig. 1. In the absence of drug these cells multiplied five to six-fold over a 3-day period. Nitrogen mustard* at 0·1 μg/ml limited cellular proliferation to about 50 per cent of the untreated cell count in this experiment. If HN2 was preincubated with medium for 12 hr before adding the medium to the cells, the drug lost its growth-inhibitory effect (Table 1). On the basis of these results, it was decided to study the effects of 0·1 μg/ml of the drug on the synthesis of protein, RNA and DNA.

Eighty-two replicate bottles were inoculated with cells, and after 2 days of growth in the normal medium, the medium was replaced with either normal medium, or medium containing HN2 at 0·1 μg/ml. The bottles were incubated for an additional 3 days at 37 °C. Cell counts were obtained daily from three bottles in each group. Different numbers of bottles were used for the chemical determinations, the contents of twenty bottles being pooled on day 1, while three bottles were sufficient on day 5.

* The nitrogen mustard used was triturated mechlorethamine hydrochloride (Merck, Sharp & Dohme). It was dissolved in water, diluted, and added to the medium immediately before use.

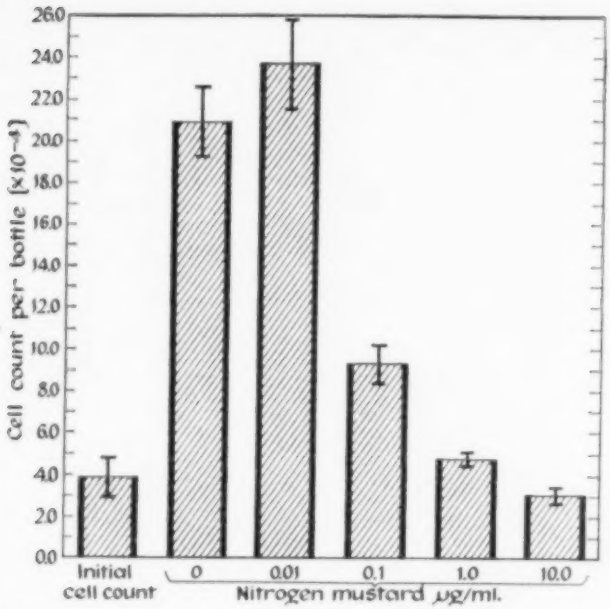

FIG. 1. Growth of fibroblasts after 3 days in varying concentrations of nitrogen mustard. Cells were inoculated into 8-oz prescription bottles, as described in the text. After 1 day, the supernatant medium was replaced with medium containing nitrogen mustard. The number of cells present in the bottles after 3 days in the experimental media was determined. The height of the bar represents the mean of of a group of three replicate bottles; standard errors of the means are also given.

TABLE 1. THE EFFECT OF PREINCUBATION OF NITROGEN MUSTARD WITH CULTURE MEDIUM ON THE GROWTH OF MOUSE FIBROBLASTS *in vitro*

Treatment	Cell count $\times 10^{-5}$
Initial inoculum	0·99 ± 0·04
Control, no drug	25·1 ± 6·9
HN2, 0·1 μg/ml	3·9 ± 0·4
1·0 μg/ml	1·6 ± 0·1
10 μg/ml	1·2 ± 0·1
HN2, 0·1 μg/ml, preincubated	19·6 ± 3·7
1·0 μg/ml, preincubated	19·8 ± 2·2
10 μg/ml, preincubated	19·6 ± 3·9

Prescription bottles were inoculated with mouse fibroblasts in Eagle's medium and on the following day, the medium was changed to one containing freshly added nitrogen mustard at the specified concentrations. Other bottles received medium containing the same concentrations of HN2, except that it was added to the medium on the previous day and allowed to incubate overnight at room temperature. Results are expressed as the mean cell count per bottle, ± standard error, obtained 6 days after the inoculation. There were three bottles in each group. The initial inoculum refers to the cell count obtained on the day after inoculation.

Fig. 2 demonstrates that the accumulation of protein and RNA in each bottle continue at about the normal rate after HN2 addition, while the increase in both cell count and DNA showed an abrupt halt after 1 day on the drug. The data are presented in terms of amounts of protein, RNA, and DNA per 10^5 cells in Table 2. There is a

large, and continuing, increase in ribose and protein per cell in the drug-treated group; at the same time these cells contain twice the normal complement of DNA, which does not increase further. The difference in the mean DNA content per 10^5 cells of the treated and untreated groups is significant at the 2 per cent level, as calculated by the Students *t*-test.

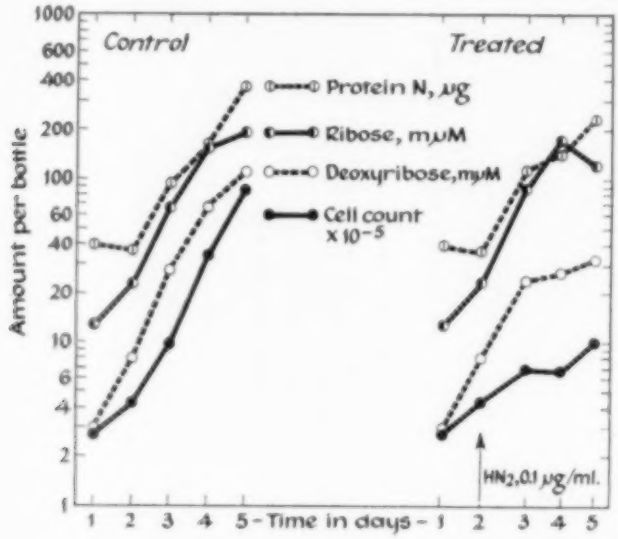

FIG. 2. Cell count, protein, ribose, and deoxyribose assays per bottle during a 5-day growth period in normal Eagle's medium and during a 3-day growth period in medium containing 0·1 μg of HN2 per ml. The cell count represents the mean of three bottles. Values for protein, ribose, and deoxyribose are the average of duplicate determinations performed on pooled bottles as described in the text.

TABLE 2. EFFECTS OF NITROGEN MUSTARD ON CELL COUNTS, RNA, PROTEIN AND DNA ACCUMULATION OF MOUSE FIBROBLASTS

Day	Untreated				HN2, 0·1 μg/ml, day 2			
	Cell count × 10^{-5}	Per 10^5 cells			Cell count × 10^{-5}	Per 10^5 cells		
		Ribose (mμmoles)	Deoxy-ribose (mμmoles)	Protein N (μg)		Ribose (mμmoles)	Deoxy-ribose (mμmoles)	Protein N (μg)
1	2·78 ± 0·30	4·7	1·1	14·2				
2	4·31 ± 0·20	5·2	1·8	8·4				
3	9·83 ± 0·90	6·8	2·8	10·2	6·86 ± 1·0	12·9	3·5	16·4
4	34·7 ± 1·0	4·4	1·9	4·7	6·78 ± 0·9	25·6	3·9	25·4
5	86·9 ± 4·0	2·2	1·3	3·4	10·0 ± 0·4	12·1	3·3	23·2
Mean ± s.e.		1·8 ± 0·3				3·6 ± 0·2		

Eighty-two replicate bottles were inoculated with cells and cell counts and chemical assays were obtained on subsequent days as described in the text. Two days after inoculation, the medium was replaced in all remaining bottles, and nitrogen mustard added to half the bottles. The medium was replaced in all bottles on day 4 with the appropriate fresh medium.

Photographs of typical normal cells, and cells from an HN2-treated culture, are given in Fig. 3. It can be seen that the drug-treated cell has a large accumulation of protoplasm and enlarged nucleoli in comparison with normal, rapidly growing cells.

Since it seemed important to determine if the doubled amount of DNA found in the drug-treated cells was a specific effect of nitrogen mustard, or was merely a reflexion of the halt in growth, cellular growth was limited by depriving a growing

TABLE 3. EFFECTS OF LEUCINE-DEPRIVATION ON CELL COUNTS, RNA, PROTEIN AND DNA ACCUMULATION OF MOUSE FIBROBLASTS

Day	Untreated				No leucine			
	Cell count $\times 10^{-5}$	Per 10^5 cells			Cell count $\times 10^{-5}$	Per 10^5 cells		
		Ribose (mμmoles)	Deoxy-ribose (mμmoles)	Protein N (μg)		Ribose (mμmoles)	Deoxy-ribose (mμmoles)	Protein N (μg)
1	2·01 ± 0·10	6·3	2·7	11·3				
2	5·13 ± 0·13	5·9	2·5	6·6				
3	10·2 ± 0·4	5·9	2·9	5·7	10·6 ± 0·3	5·7	2·9	4·7
4	25·5 ± 0·8	5·5	2·4	3·1	15·4 ± 0·4	6·5	2·9	4·6
5	63·5 ± 2·6	3·3	2·0	2·9	18·1 ± 1·4	6·5	1·8	3·6
Mean ± s.e.			2·5 ± 0·2				2·5 ± 0·4	

One hundred and three replicate bottles were inoculated with cells and cell counts and chemical assays were obtained on subsequent days as described in the text. Two days after inoculation, the normal medium was replaced with a fresh medium containing dialysed serum, and no leucine. Half the bottles (the untreated group) were supplemented with L-leucine at the usual concentration (0·1 millimolar).

culture of an essential amino acid, leucine. The results of this experiment appear in Table 3. It is apparent that when the amount of leucine in the culture medium is restricted, the cells shortly fail to grow; the cell count fails to increase normally, DNA synthesis halts, and RNA and protein synthesis also cease. Of more direct interest, however, is the finding that the DNA content per cell of cultures deprived of leucine is essentially the same as the DNA content of normal, rapidly growing cells.

Additional experiments with nitrogen mustard have confirmed the effects previously described. It should be noted, however, that the concentration of drug employed is critical. At concentrations lower than 0·1 μg/ml, little or no effect is observed. At higher concentrations (0·2 or 0·3 μg/ml), protein and RNA synthesis are also depressed, and cell lysis eventually occurs. Under these conditions, however, the DNA content per 10^5 cells remains higher than normal, at about 3·6 mμmoles of deoxyribose. The mean DNA content of normal, rapidly growing cells has been found, in later experiments, to be quite constant at about 2·4 mμmoles per 10^5 cells. The low value of 1·8 mμmoles reported in Table 2 (untreated cells) has not been seen in subsequent experiments, although the DNA content of HN2-treated cells has been observed (three experiments) to remain at 3·6 mμmoles.

The decline in protein content per cell, as log phase growth proceeds (Table 2 and, more typically, Table 3, untreated cells), is quite regularly observed and has been discussed by others.[9]

DISCUSSION

The data presented in Table 1 indicate that nitrogen mustard, and not one of its degradation products, is responsible for the growth-inhibitory effect reported here. Furthermore, it is clear that HN2 is not reacting with some component of the medium to produce its effect. The effects of low concentrations of nitrogen mustard, therefore, are a rapid cessation of cell division and DNA synthesis, but RNA and protein synthesis continues for several days at about the normal rate. Cells inhibited with HN2, therefore, had several-fold the normal complement of RNA and protein, and were much larger than normal, rapidly growing cells. While DNA synthesis showed an abrupt halt shortly after HN2 addition, these inhibited cells nevertheless had up to twice the normal amount of DNA found in a rapidly growing cell. The increased amount of DNA per cell after HN2 addition is interpreted to mean that these cells synthesize DNA up to the pre-mitotic amount, and then DNA synthesis ceases. Cells whose growth was limited by leucine-deprivation, on the other hand, had the same amount of DNA per cell as was found in a rapidly growing culture. These effects of HN2 are similar to the effects of X-radiation on mouse fibroblasts, as described by Whitfield and Rixon.[14] These authors observed a "piling up" of DNA at the pre-mitotic amount, and little effect on protein and RNA synthesis. Caspersson *et al.*, in a microspectrophotometric study of single ascites cells after X-radiation, also found an accumulation of cells containing the pre-mitotic amount of DNA.

This evidence suggests that nitrogen mustard does not interfere with the functional aspects of DNA metabolism, at least insofar as they are reflected in RNA and protein synthesis. It also suggests that DNA replication occurred, giving rise to cells containing the pre-mitotic amount of DNA, but final separation of the DNA into daughter chromosomes and cellular division no longer occurred. Undoubtedly the cells will develop into typical "giant cells", widely reported[16] to occur after nitrogen mustard treatment or radiation. Cobb[16] has specifically examined the cytological effects of HN2 on mammalian cells growing *in vitro*, and readily observes giant cell formation. She also reports observing "a suppression of mitosis and aberrant and sticky chromosomes".

If current theories of DNA replication,[17] involving unwinding of a parent polynucleotide helix with the simultaneous formation of two daughter helices, are correct, then it is hard to see how a simple cross-linking of DNA molecules could lead to the observed results. If DNA strands were cross-linked, and therefore unable to separate, one would expect a complete cessation of DNA synthesis as well as cell division. On the other hand, if cross-linking of chromosomal material occurred at an early prophase stage of division, such that chromatids perhaps could not condense or separate, one might expect to see a pre-mitotic value of DNA, as was actually observed. Such a scheme of cross-linking might possibly involve DNA–DNA interaction, as originally proposed by Goldacre *et al.*,[1] and Alexander and Lett.[2] However, it seems equally possible, and perhaps more likely, that DNA-nucleoprotein cross-linking could be responsible for the observed results. In fact, any interference with an early step in the mitotic process could give rise to these effects.

Acknowledgement—We would like to thank Prof. Frederick A. Fuhrman and the staff of the Max C. Fleischmann Laboratories of the Medical Sciences for their invaluable assistance during the course of these experiments. This work was partially supported by a grant (CY2797) from the National Cancer Institute to Prof. Avram Goldstein.

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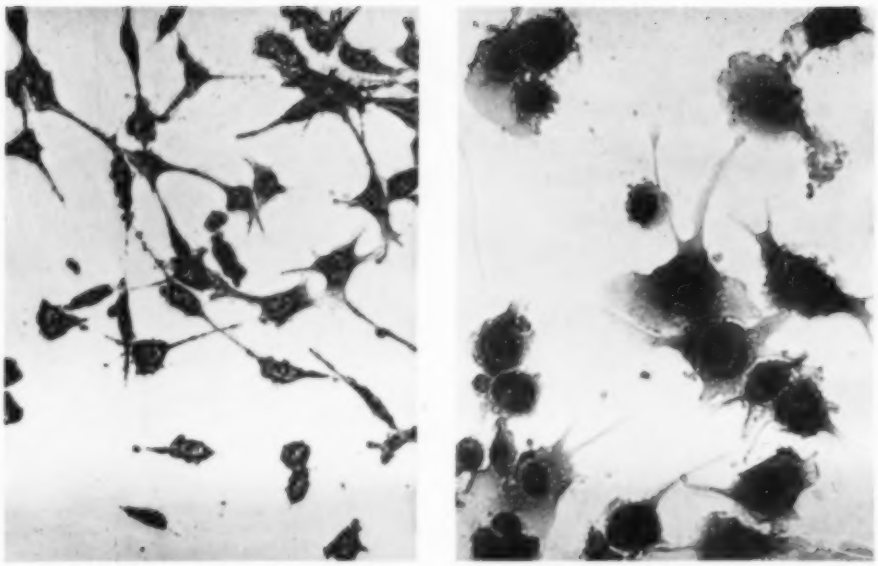

FIG. 3. Left: Normal fibroblasts, growing on microscope slides in Blake bottles. After 5 days of culture, the slide was removed, rinsed with a buffered saline solution and fixed in methanol. The slide was lightly stained with Giemsa solution. × 53·5.

Right: Same as above, but incubated for the final 2 days in medium containing 0·1 μg of HN2 per ml.

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THE MECHANISM OF STIMULATION OF AEROBIC FERMENTATION IN YEAST BY A QUATERNARY AMMONIUM DETERGENT*

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Abstract—Of the various metabolic effects produced in yeast cells by the cationic detergent, benzalkonium, the most pronounced is the inhibition of the pathway of respiration of two-carbon compounds. Less sensitive is the fermentation of glucose and the respiration of three-carbon substances. Because glucose is normally respired via two pathways, the formation of two-carbon substances and the direct oxidation of pyruvate, benzalkonium disturbs the normal pattern of its metabolism. The R.Q. is markedly elevated. The "extra CO_2" production is paralleled by an appearance of ethyl alcohol. This action of benzalkonium on glucose can be largely duplicated by over-loading the system through addition of two-carbon compounds such as ethyl alcohol, acetaldehyde or acetate, or by excessive concentrations of glucose itself. The detergent also causes an increase in the total amount of O_2 consumed for a given amount of substrate and a change in the R.Q. for the respiration of alcohol or acetaldehyde. These actions are related to the inhibition of carbohydrate assimilation.

INTRODUCTION

BECAUSE of their importance as antiseptic agents, the action of quaternary ammonium detergents on microbial cells has been much investigated. Yeast cells have also served as convenient test organisms. A variety of inhibitory effects has been observed. Of these, the inhibition of growth is most sensitive, followed by the inhibition of respiration and finally by the lysis of the cells. The three inhibitory effects are apparently quite independent of each other.[1] Recently, the lytic effect has been quantitated in terms of a loss of K^+ from the cells, an inhibition of anaerobic glycolysis and the ability of cells to decarboxylate pyruvate under anaerobic conditions.[2]

In addition to the general inhibitory effects, a stimulation of respiration was observed at sharply defined, but very low concentrations of Zephyran.[1‡] More recently Scharff and Beck[3] have shown that certain concentrations of this agent induce in baker's yeast a substantial increase in aerobic fermentation, accompanied by only a small increase of oxygen consumption and of hexose uptake. A true inhibition of the Pasteur effect seems to be involved, as opposed to the simple switch-over from respira-

* This study is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

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‡ Zephyran is a mixture of high molecular weight alkyl-dimethylbenzyl-ammonium chlorides, commonly called benzalkonium chloride. For convenience, this preparation will be denoted in this paper as benzalkonium, or in the figures as B.Z.

tion to glycolysis that occurs with respiratory inhibitors such as cyanide and carbon monoxide.

The action of the cationic detergents involves at least in parts a binding at the cell surface. The lytic action itself implies a breakdown of the cell membrane. Furthermore, the lytic action on yeast can be prevented by uranyl ions[4, 5] which do not penetrate, but combine firmly with anionic groups of the cell surface.[6] The possibility of metabolic effects by agents acting at the cell surface is supported by evidence[7–9] pointing to the location of glycolytic enzymes in the periphery of the cell. In view of these facts, it seemed profitable to investigate further, the action of benzalkonium on the metabolism of yeast using a variety of substrates, in an attempt to localize the exact point of action in the metabolic sequence.

EXPERIMENTAL AND RESULTS

Fresh baker's yeast (Standard Brands, Incorporated) was used in all experiments. The cells were thoroughly washed several times by suspending in water and centrifuging. The speed of the centrifugation was low, so that colloidal material and cell debris was discarded. After aeration for several hours the cells were centrifuged and resuspended in water or buffer. The final concentration of the suspension was checked by photometric measurement of its turbidity. The cells were incubated at 26 °C in the presence of appropriate additions, and air or pure nitrogen, respectively, were bubbled through the suspension to achieve mixing and aerobic or anaerobic conditions. For chemical determinations samples were taken out, centrifuged at high speed (10,000 g and analyses were carried out on the supernatant.

Ethanol was determined by the alcohol–dehydrogenase method according to Bücher and Redetzki.[10]

Glucose was determined by the glucose oxidase method, according to a modification of the method of Saifer *et al.*[11]

Potassium was determined by means of the flame photometer (Beckman attachment with a model DU Beckman spectrophotometer).

Buffer solutions. Triethylamine-succinate-tartrate buffer at pH 4·5 was used in all experiments where the use of buffer is indicated.[12]

Gas exchanges were measured by standard Warburg manometry with air or nitrogen, respectively, in the gas phase.

The effects of benzalkonium were found to be proportional to the concentration of cells, at least in the ranges used in the present experiments (from 10 to 40 mg cells wet wt./ml of suspension). For this reason, the amounts of detergent used in particular experiments are always expressed in terms of μg/mg wet weight of cells. The concentration of cells is given for each experiment so that the concentration of detergent can be readily calculated.

One of the striking effects of benzalkonium is the stimulation of aerobic fermentation.[3] In studying this response, it was observed that even in untreated baker's yeast the rate of aerobic fermentation was very high, and that a primary factor is the glucose concentration (Fig. 1). At levels below 0·01 M, the aerobic fermentation (measured as "extra CO_2" above R.Q. of 1·0) was minimal, but at relatively high concentrations of glucose, the rate of "extra CO_2" production was almost half as high as the rate of respiration (R.Q. of 1·5). The glucose concentration chosen for most experiments was

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0·025 M, sufficiently high to give a maximal rate of respiration initially, but sufficiently low to induce minimal aerobic fermentation.

With the lowest effective concentration of detergent (1·5 μg/mg wet wt. of yeast) the rate of respiration of glucose was not altered during the first 2 hr, but, in contrast to the control, remained linear for the subsequent 2 hr. Consequently, the

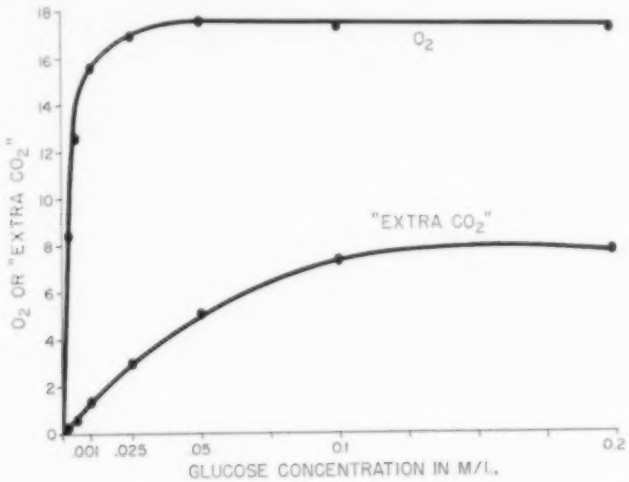

FIG. 1. The dependence of the rate of respiration and of the rate of "extra CO_2" production on the glucose concentration; 20 mg/ml yeast wet weight in 0·02 M triethylamine–succinate–tartrate buffer pH 4·5, containing 0·002 M KCl and the indicated concentrations of glucose.

total O_2 consumption was considerably increased (Fig. 2). In contrast to the unaltered rate of respiration, the initial rate of CO_2 production increased from 19·5 μl/mg per hr to 40·0 μl/mg per hr, indicating that aerobic fermentation was enhanced (in Fig. 2 the effects are expressed in terms of the "extra CO_2" over an R.Q. of 1·0). With 1·5 μg/mg of detergent the "extra CO_2" production was considerable, but transient. It reached a peak in 30 min and thereafter had a negative value, so that by 6 hr the "extra CO_2" was virtually zero. The action of the detergent can also be expressed in terms of the R.Q. which was considerably greater than 1·0 during the first 30 min, and was less than 1·0 thereafter.

With a higher concentration of detergent (3 μg/mg of yeast) the rate of respiration was inhibited to the extent of about 50 per cent, but, in contrast to the control, the rate was nearly constant for 6 hr, rather than for only 2 hr. The action on aerobic fermentation was more prolonged. With 6 μg/mg of yeast, the respiration was depressed to the endogenous level and little aerobic fermentation was observed. The endogenous respiration itself is insensitive to these concentrations of detergent.

Anaerobic CO_2 production was not stimulated by the detergent, but only inhibited. At 3 μg/mg, the inhibition was 33 per cent and at 5 μg it was 82 per cent.

The dramatic changes in the relationship between O_2 consumption and CO_2 production, induced by the detergent, first a production of "extra CO_2" and then a decrease in "extra CO_2", suggested that the response might be due to the accumulation

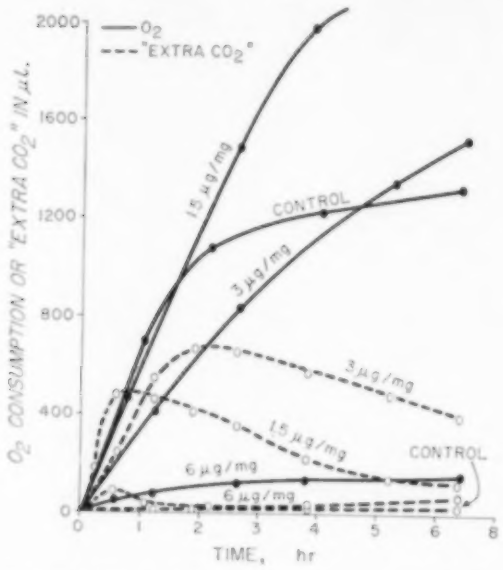

FIG. 2. The effect of benzalkonium on the respiration and "extra CO_2" production from glucose; 20 mg/ml of yeast in distilled water with 0·025 M glucose and benzalkonium as indicated.

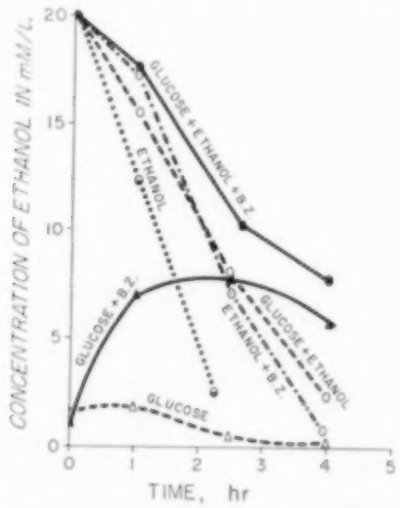

FIG. 3. The influence of benzalkonium on the alcohol content of the medium with glucose or alcohol or mixtures as substrates; 20 mg/ml yeast in distilled water with 0·025 M glucose and/or ethanol and 3 µg/mg benzalkonium where indicated.

of a metabolic intermediate which is in a reduced state relative to glucose, followed at a later time by the oxidation of this intermediate. Transient accumulations of ethyl alcohol have been reported in yeast presented with high concentrations of glucose.[13] A series of experiments was performed with glucose and ethyl alcohol alone and in mixtures (Fig. 3). With glucose alone (concentration of 0·025 M) little alcohol was

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found. With glucose plus detergent, however, a large output of alcohol was found which was parallel to the "extra CO_2" shown in Fig. 2. With alcohol added initially, its rate of disappearance was decreased by about 40 per cent by either the addition of detergent or of glucose and was decreased about 60 per cent if both glucose and detergent were present.

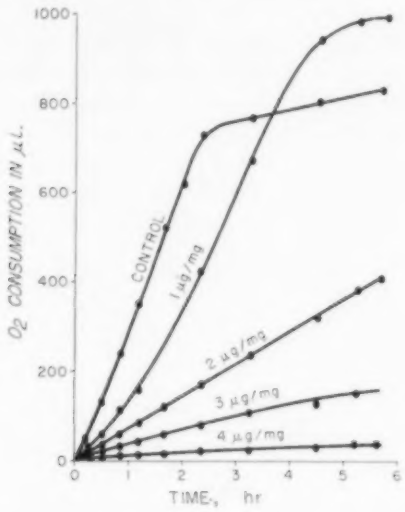


FIG. 4. The effect of benzalkonium on the respiration of acetate; 20 mg/ml yeast in 0·02 M buffer, pH 4·5 with 0·025 M acetate, and indicated concentrations of benzalkonium.

TABLE 1. INFLUENCE OF BENZALKONIUM ON THE RESPIRATION OF VARIOUS SUBSTRATES

(Yeast, 20 mg/ml in water; substrates, 0·03 M; benzalkonium, 3 μg/mg of yeast.)

Substrate	O_2		
	Control	B.Z.	Inhibition %
Pyruvate	8·8	5·9	33
Lactate	8·8	5·9	33
Glucose	18·0	8·0	55
Ethanol	19·5	4·5	77
Acetaldehyde	13·4	2·7	80
Acetate	13·4	1·5	89
Glucose and acetate	19·5	1·5	92

The rates are in μl/mg per hr for the first hour.

Eaton and Klein[13] and Holzer[14] suggested that a fraction of glucose is respired via glycolysis and subsequent oxidation via the two-carbon pathway and the Krebs cycle. The accumulation of alcohol in the presence of benzalkonium and the inhibition of its oxidation would suggest that the agent acts somewhere in the two-carbon pathway. A series of tests was carried out using a variety of substrates. A typical set of data is

given in Fig. 4 for acetate. As in the case of glucose, the rate of respiration was reduced, but the rate was maintained for a longer period than in the case of the control. Comparative data for a variety of substrates are given in Table 1.* The respiration (O_2 consumption) of the two-carbon compounds was the most sensitive to the agent in the order: acetate, acetaldehyde, ethanol (from 89 to 77 per cent inhibition). The respiration of the three-carbon compounds, lactate and pyruvate, was much less sensitive (33 per cent inhibition), a finding which is in line with the fact that the metabolism of these substances does not go via the two-carbon pathways.[15] The respiration of glucose (O_2 consumption) was intermediate in sensitivity (55 per cent inhibition). However, the disappearance of glucose, and the anaerobic CO_2 production were of lesser sensitivity (from 32 to 33 per cent inhibition). These observations are compatible with the concept that part of the respiration of glucose proceeds via the two-carbon pathway and that this component is most sensitive to the detergent, whereas the glycolytic path is relatively insensitive.

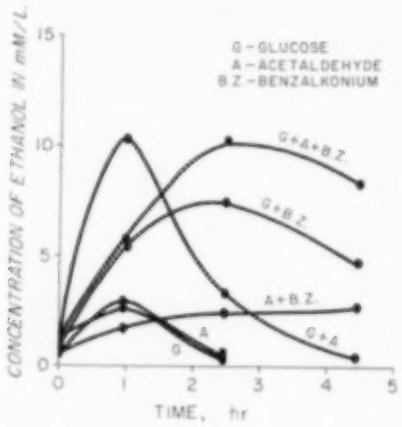


FIG. 5. The effect of benzalkonium on alcohol production from glucose, from acetaldehyde or from both substrates; 20 mg/ml yeast in distilled water with 0·025 M glucose and/or acetaldehyde and 3 μg/mg benzalkonium where indicated.

If the detergent blocks at the two-carbon level, its action on glucose metabolism should be imitated by "loading" the two-carbon pathway with exogenous two-carbon substrates. A series of experiments were therefore carried out with glucose, two-carbon substrates, and detergent, alone, and in mixtures. The data of Fig. 5 indicate that acetaldehyde, like the detergent, induced a large yield of alcohol from glucose. The effect with acetaldehyde was transient because this substance is used as a substrate, whereas the effect with detergent was prolonged. That acetaldehyde does not itself act as a major source of the alcohol is indicated by the fact that glucose is essential. Acetaldehyde alone, or together with detergent, gave only a low yield of

* The organic acids are respired only if free acid is present in the medium.[20] Because the pH tends to rise during their metabolism, it is necessary to use buffers. The buffer system chosen was triethlamine–succinate–tartrate.[12] Although cations in general have a protective action against the cationic detergents (see later section) the effect of triethlamine in the concentration used was small and did not change the relative effects on the different substrates.

alcohol. A similar response is also found in terms of "extra CO_2" production (Fig. 6). With acetaldehyde as a substrate, less CO_2 was produced than O_2 consumed (theoretical R.Q. is 0·84). With glucose alone, a small surplus of CO_2 was found. With a mixture of glucose and acetaldehyde, however, the surplus of CO_2 was considerably but temporarily increased. The detergent produces a similar but more permanent increase.

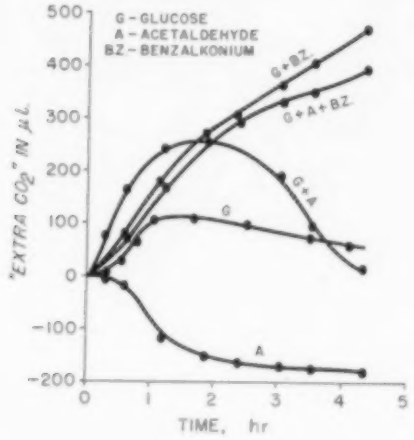


FIG. 6. The effect of benzalkonium on the "extra CO_2" production from glucose, from acetaldehyde, or from both substrates. Identical to Fig. 5.

Acetate also behaved like acetaldehyde, increasing the output of alcohol and of "extra CO_2" from glucose.*

In Table 1, it was demonstrated that benzalkonium in appropriate concentrations inhibited the respiration of all of the substrates tested. In addition to this inhibition, the relationship of O_2 consumption to CO_2 production was altered. The results, in the case of ethyl alcohol, are given in terms of the R.Q. (Fig. 7). Although the theoretical value is 0·67, the control values were considerably lower (about 0·3) for about 1 hr, and somewhat higher (about 0·8) thereafter. However, at the end of 5 hr, the average R.Q. was 0·59, reasonably close to theoretical. The rate of respiration was relatively linear for the whole period, but the CO_2 production lagged initially, and finally caught up. Apparently alcohol is initially converted in part to a metabolite in a higher state of oxidation which accumulates for a time, but which is finally respired. With benzalkonium present, the pattern was quite different; the R.Q. started at 1·08 and gradually drifted to about 0·7. The average R.Q. for the 5-hr period was greater than theoretical. It must be kept in mind, however, that the rate of respiration in this case was only about 30 per cent of normal, so that the endogenous metabolism represents about 30 per cent of the total respiration (compared with less than 10 per cent in the control).

The pattern for R.Q.'s for acetaldehyde was similar. The theoretical value is 0·84, but during the first hour, the observed value was less than 0·7. In the presence of

* However, the inhibitory effect of detergent on the respiration of glucose was markedly enhanced in the presence of acetate, increasing from 55 to 92 per cent (Table 1). None of the other two- or three-carbon compounds had this effect, nor did acetate have any such effect in the absence of detergent.

detergent, the value was slightly over 1·0. In the case of acetate, on the other hand, the observed R.Q. was close to the theoretical (1·0) during the whole period of metabolism and the addition of detergent had little effect.

The action of benzalkonium is reported to involve a binding to anionic sites on the cell surface.[4] For this reason, cations in the medium will protect by competing for the binding sites, provided that they are added prior to, or at the same time as the detergent.[20] In the present studies cations were found to exert a strong protective action

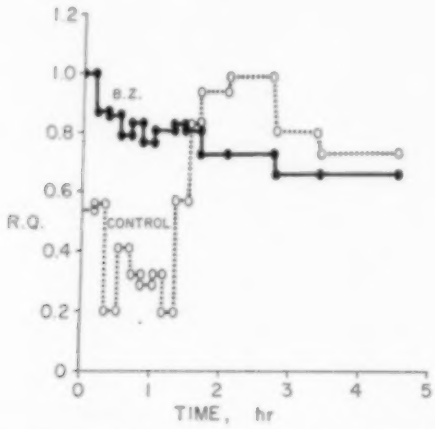


FIG. 7. The effect of benzalkonium on the R.Q. with alcohol as a substrate; 20 mg/ml yeast in 0·02 M buffer, pH 4·5 with 0·025 M ethanol and 3 μg/mg benzalkonium where indicated.

TABLE 2. PROTECTIVE ACTION OF Ca^{2+} AGAINST "EXTRA CO_2" PRODUCTION INDUCED BY BENZALKONIUM

(The yeast concentration was 20 μg/ml; glucose, 25 mM; benzalkonium, 3 μg/mg; and Ca^{2+} (as $CaCl_2$ adjusted to pH 4·5), 0·1 M.)

Time (hr)	Control	B.Z.	B.Z.	Ca^{2+}
0	0	0	0	0
0·5	20	120	150	20
1·0	20	280	320	20
1·0	No Additions		Ca added	B.Z. added
1·5	24	420	460	24
2·0	30	520	560	30
3·0	40	640	560	80
4·0	40	700	460	60
5·0	40	700	320	40
6·0	40	620	160	30

if added before the detergent, a slow but definite reversal if added after the detergent. For example, 0·1 M Ca^{2+} gave almost complete protection against "extra CO_2" production when added 1 hr before benzalkonium (Table 2). When the order of addition was reversed (B.Z. added 1 hr prior to Ca^{2+}), the action of the detergent was modified

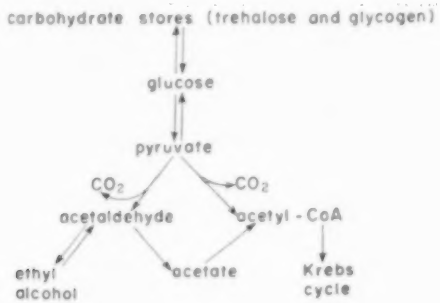
only after a delay period of from 1 to 2 hr. Five hours after the addition of Ca^{2+}, much of the "extra CO_2" had disappeared.

The inhibition of respiration induced by the detergent could also be modified by cations. If the cations were added before the detergent, complete protection could be afforded, but if the detergent was added first, the effect was only partially reversed and only after a delay of from 1 to 2 hr. Washing with solutions containing bivalent had little additional effect. No extensive tests were carried out with different cations, but from the available data, the relative efficacy of protection was in the order Na^+, K^+, triethylamine$^+$ < Mg^{2+} < Ca^{2+} < UO^{2+}.

DISCUSSION

Among the multiple effects of benzalkonium on metabolism are: (1) the increase in total O_2 consumption from a given quantity of substrate; (2) the inhibition of respiration of various substrates; (3) the inhibition of anaerobic metabolism of glucose; (4) the stimulation of alcohol production and of "extra CO_2" from glucose; and (5) the changes in R.Q. for alcohol and acetaldehyde.

The interpretation of these phenomena and their interrelationships should be consistent with our knowledge of the pathways of glucose metabolism in yeast. A current picture is one summarized by Holzer.[14] Added to his scheme is the pathway of assimilation (formation of carbohydrate stores) which may account for from 20 to 40 per cent of substrate utilization, including that of two- or three-carbon compounds.[16, 17]



The relative importance of the two parthways of pyruvate degradation depends on its intracellular concentration; at low levels the pyruvic oxidase (right) is saturated first, due to its lower K_M; at higher levels the pyruvate overflows into the decarboxylative pathway (left), which is joined again to acetyl-CoA through the dehydrogenases and the aceto-CoA kinase. This picture is in agreement with the known facts and notably with the findings that the rate of fermentation is parallel to the intracellular concentration of pyruvate.[18, 19] It is also consistent with the appearance of aerobic fermentation only at higher glucose concentrations in the medium (Fig. 1) and with the previously mentioned results of Eaton and Klein[13] who found, using glucose-3, 4-^{14}C, that most of the label was in the CO_2 evolved initially, i.e., during the phase when ethanol temporarily accumulates in the medium.

It can be predicted from the above scheme that aerobic fermentation, that is, accumulation of ethanol and "extra CO_2" will occur whenever the rate of the

carboxylase reaction exceeds the rate of oxidation of the two-carbon intermediates. Three conditions producing this imbalance have been demonstrated:

(a) a high external concentration of glucose (Fig. 1);

(b) overloading of the oxidative system with exogenous two-carbon intermediates (Figs. 3, 5, 6);

(c) inhibition of the oxidation of one of the two-carbon intermediates by benzalkonium (Figs. 2, 3, for example).

In all cases, the effect is transient. After the glucose concentration is reduced to levels which cannot maintain maximal rates of respiration, the accumulated alcohol is respired and in consequence, the "extra CO_2" disappears.

This explanation of the benzalkonium-induced alcohol accumulation or "extra CO_2" production is consistent with the fact that the respiration of two-carbon substances is more sensitive to benzalkonium than is the respiration of glucose or of three-carbon compounds, or the fermentation of glucose. It is not possible to pinpoint the exact site of inhibition. The reaction alcohol $\leftrightharpoons$ acetaldehyde is not blocked, or alcohol would not accumulate. But either the reaction acetaldehyde to acetate, or acetate to acetyl-CoA, must be more sensitive to benzalkonium than any reactions in the glycolytic sequence or in the pyruvate-to-Krebs cycle-to-cytochrome sequence. With higher concentrations of the agent, reactions in the glycolytic path and in the respiratory pathway are also blocked.

The increase in total O_2 consumption induced by benzalkonium from each of the substrates tested, suggests a diminished assimilation (formation of carbohydrate stores). A few direct measurements of the changes in the total intracellular carbohydrates using the anthrone method[20] confirmed this suggestion. The inhibition of the assimilation process also provides an explanation for the R.Q. patterns with alcohol (Fig. 7). In the control, the initial low values (about 0·4) probably represent formation of carbohydrate from alcohol. The higher values in the presence of benzalkonium are consistent with the suggestion that assimilation is blocked or that carbohydrate stores are mobilized. The pattern with acetaldehyde is similar but the changes are smaller because the acetaldehyde is already at a higher state of oxidation than is alcohol. With acetate, no changes in R.Q. were observed because it is in the same state of oxidation as carbohydrate.

Basic dyes behave in some respects like benzalkonium. They bind on anionic groups of the cell surface and produce an irreversible effect from which protection is afforded by inorganic cations.[22–24] The basic dyes cause disruption of the cell membrane as a permeability barrier so that intracellular constituents such as K^+ leak out and the cells are stained. The response is all or none for individual cells.[24] Benzalkonium, at high concentrations, can produce a similar, all-or-none, breakdown of the cellular membrane as measured by loss of K^+, or staining, or release of carboxylase.[2] However, the reported specific actions on the metabolism of two-carbon compounds are apparently independent of the lytic action itself. First, with basic dyes, which also cause lysis, the "extra CO_2" production was not observed.[22, 23] Second, the effects on endogenous respiration associated with the lytic action, require higher concentrations of detergent than those on the exogenous metabolism.[5] Third, the concentrations of detergent required for the lytic effect are higher than those which produce "extra CO_2". Scharff and Maupin,[2] using the same yeast and detergent as in the present experiments,

studied the release of K^+ in detail. The lytic effect was minimal below a concentration of benzalkonium of 4 to 5 μg/mg of yeast, but increased rapidly with higher concentrations. In the present experiments no K^+-loss was induced by detergent at concentrations of 1·5 or 3·0 μg/mg, but K^+-loss was 30 per cent in a few hours at 6·0 μg/mg (in good agreement with Scharff and Maupin[2]). In view of the fact that the metabolic effects reported in this paper predominated at concentrations of 1·5 and 3·0 μg/mg, they are presumably independent of the lytic effect.

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INFLUENCE OF β-PHENYL*ISO*PROPYLHYDRAZINE ON THE ABILITY OF BLOOD PLATELETS TO RETAIN 5-HYDROXYTRYPTAMINE

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Abstract—Platelet-rich plasma of rabbits, rats and humans was incubated under various treatments and the 5-hydroxytryptamine (5HT) content of separated platelets was analysed per ml of original plasma volume.

More 5HT was found after the incubation when monoamine oxidase (MAO) inhibitor, β-phenyl*iso*propylhydrazine (PIH) was present in concentrations of 10^{-4} or 10^{-3}M. Incubation in nitrogen instead of air had similar effect. At higher concentrations (10^{-3} or 10^{-2}M, according to the species) PIH produced a marked 5HT depletion. Iproniazid, as well as isoniazid, which is not a MAO inhibitor, decreased the platelet 5HT when tested on rat platelet-rich plasma at 10^{-2}M.

In rabbit's platelets PIH (from 10^{-5} to 10^{-3}M) partly inhibited the release of 5HT produced by tetrabenazine. A nitrogen atmosphere had the same effect and PIH in addition to nitrogen offered no further inhibition. Isoniazid 10^{-3}M was ineffective. In suitable concentrations PIH also had an inhibitory action on the 5HT release from platelets of rats and humans. The inhibition of 5HT depletion is assumed to be connected with the inhibition of the platelets ability to inactivate their 5HT.

RAUWOLFIA alkaloids and tetrabenazine cause a decrease of 5-hydroxytryptamine (5HT) content in various tissues including the blood platelets.[1–3] After treatment with monoamine oxidase (MAO) inhibitor, iproniazid, this depletion is, at least partly, inhibited in brain tissue.[4, 5] A further proof for this inhibition were experiments, where rabbit's platelet-rich plasma was incubated *in vitro* with reserpine. More 5HT was left in the platelets if, in addition to reserpine, iproniazid was present in the plasma.[6]

This paper describes experiments which were designed to study especially the effect of another MAO inhibitor, β-phenyl*iso*propylhydrazine,[7] on the 5HT decrease in platelets. Because platelets of rabbit inactivate their 5HT more readily than those of man and rat,[8] the platelets of all these three species have been studied. As the agent releasing 5HT, tetrabenazine methane sulphonate was used, because it is soluble in saline. The mode of action of this benzoquinolizine derivative is related to that of reserpine.[3, 9]

METHODS

Arterial blood was taken from a carotid artery of male rabbits (2·3–3·0 kg) and from the abdominal aorta of male rats (200–300 g) in ether anaesthesia. Human venous blood was collected from a cubital vein. Blood was immediately mixed with $\frac{1}{9}$ vol. of 1% disodium ethylenediaminetetra-acetate and 0·1% heparin in saline. Rabbits were heparinized (5 mg/kg intravenously) immediately before bleeding.

After centrifuging the blood at about 200 g for 5 min the platelet-rich plasma was withdrawn. The remaining blood was then again centrifuged at least once more, and the platelet-rich plasma was removed after each spin, and the procedure repeated once or more. This plasma (sample volume 2–3 ml) was incubated with tetrabenazine methane sulphonate (Nitoman®, F. Hoffman—La Roche & Co, Basel) under gentle shaking for 3 hr in 25 ml Erlenmeyer flasks at 37 °C in air. Some experiments were carried out in an atmosphere of nitrogen. β-Phenyl*iso*propylhydrazine hydrochloride (Catron®, Lakeside Laboratories, Inc. Milwaukee, Wisc.), iproniazid phosphate (Marsilid®, F. Hoffmann La Roche & Co. Basel) and isoniazid (Tubilysin®, Orion Oy, Helsinki) were added to the plasma 15 min before tetrabenazine. The final concentrations per ml of plasma of all substances are reported in the text. Solutions were prepared in saline and the volume of tetrabenazine solution used was 0·1 ml and that of the others 0·2 ml per ml of plasma. Solvent alone was added to the controls.

After incubation the platelets were separated by centrifugation at about 2000 g for 20 min at room temperature and their 5HT content was measured by a spectrophotofluorometric method.[10] Platelet counts were made in the phase contrast microscope.[11] All the experiments were carried out in siliconized glassware. The standard 5HT was used as creatinine sulphate (F. Hoffmann—La Roche & Co., Basel), but the doses are given in terms of the base. Standard error of the mean is given to indicate the distribution of values. Student's *t*-test was used to estimate the significance of the differences.

RESULTS

Incubation without tetrabenazine

The effect of incubation with PIH, iproniazid, isoniazid and atmosphere of nitrogen are reported in Table 1. The values are in per cent of the corresponding control figures obtained from the platelets of the same plasma.

TABLE 1. 5HT FOUND IN BLOOD PLATELETS PER ML OF PLATELET-RICH PLASMA AFTER INCUBATION WITH β-PHENYL*iso*PROPYLHYDRAZINE, IPRONIAZID AND ISONIAZID

P-values are given when they are less than 0·05 and it indicates that mean of the individual differences between control and experimental group is significantly different from zero.

Atmosphere during incubation	5HT μg/ml	5HT % of the corresponding controls					
	Controls	β-phenyl*iso*propylhydrazine M 10^{-5}	10^{-4}	10^{-3}	10^{-2}	Iproniazid M 10^{-2}	Isoniazid M 10^{-2}
				Rabbit			
Air	10·1(7)* ± 0·8†	98(4) ± 1	101(4) ± 1	106(7) ± 2	26(4) ± 5		
				$P<0·05$	$P<0·02$		
Nitrogen	16·2(4) ± 1·5			96(4) ± 8			
				Rat			
Air	3·3(12) ± 0·3	100(3) ± 6	109(6) ± 3	77(8) ± 3	16(3) ± 3	70(5) ± 6	53(4) ± 13
			$P<0·05$	$P<0·01$	$P<0·02$	$P<0·01$	$P<0·001$
				Human			
Air	0·17(8) ± 0·06		123(5) ± 13	92(6) ± 18	36(5) ± 8		
					$P<0·05$		

* Number of animals
† Standard error of the mean

In *rabbit* platelets PIH in concentrations of 10^{-5} and 10^{-4}M did not alter the 5HT content of platelets but at 10^{-3}M the amount of 5HT was always higher when compared to the control value. Ten times higher concentration of PIH had a marked opposite effect and only one-quarter of the amine was left in the platelets during 3 hr incubation. This 5HT releasing effect of PIH was time-dependent as illustrated by the following example: 5HT left after incubation with PIH 10^{-2}M for $\frac{1}{2}$ hr, 90 per cent; $1\frac{1}{2}$ hr, 60 per cent; 3 hr, 36 per cent.

In four experiments the platelet-rich plasma was incubated in nitrogen atmosphere. After the nitrogen incubation the mean control value was significantly ($P<0{\cdot}02$) more than the control value obtained after incubation in air. In nitrogen, PIH did not further increase the amount of 5HT. Isoniazid at 10^{-3}M in four experiments had no effect on the platelet 5HT.

When *rat* platelet-rich plasma was incubated with PIH at 10^{-4}M there was more 5HT in the platelets than in the controls and in those which were treated with 10^{-5}M of PIH. Also in the rat platelets a decrease of 5HT was demonstrated but a concentration only of 10^{-3}M was required to produce it.

Iproniazid and isoniazid were also tested at 10^{-2}M and they both produced a decrease of 5HT of about equal degree but their effect was weaker than that of PIH. 5HT released from platelets by PIH or iproniazid could be found in the platelet-free plasma both in rats and rabbits.

In *human* platelets PIH produced changes which were more similar to those found in rat than those found in rabbit platelets. PIH 10^{-4}M increased the 5HT yield but 10^{-2}M lowered it.

Incubation with tetrabenazine

The results are shown in Fig. 1, which gives decrease in per cent from the corresponding normal or PIH-treated control value.

In *rabbit* platelets all PIH concentrations tested significantly inhibited the release of 5HT by tetrabenazine. Incubation in nitrogen had a marked inhibitory effect on the 5HT decreasing action of tetrabenazine. Addition of PIH did not further reduce the effect of tetrabenazine under these conditions.

In four experiments with 10^{-3}M of isoniazid tetrabenazine released $50{\cdot}6 \pm 5{\cdot}5$ per cent of 5HT. In the same plasmas without isoniazid the value was $52{\cdot}4 \pm 7{\cdot}0$ per cent.

At the concentration of 10^{-4}M PIH had no inhibitory effect on the 5HT release in *rat* platelets. However, also here a significant inhibition was demonstrated at ten times higher concentration of PIH.

In *human* platelets, 10^{-3}M of PIH had a significant inhibitory effect and there was an indication that 10^{-4}M also had some effect on the 5HT release.

Biological method: pH of the platelet-rich plasma; platelet counts

Before employing the spectrophotofluorometric method, a number of experiments with rabbit platelets and some with rat platelets were made by using the rat stomach method[12] for 5HT estimation. On the whole the results were in agreement with those obtained by chemical means and reported above.

During the incubation of platelet-rich plasma in air the pH rose to about 8·0. The saline solutions of PIH, isoniazid and iproniazid are acid but the buffering capacity of plasma is high and only with the concentrations of 10^{-2}M some lowering in plasma

pH was regularly seen. This was most marked with iproniazid and least with PIH. Further experiments with rabbit and rat platelet-rich plasma not included in Table 1 have shown that the results are similar if the MAO inhibitor solutions are neutralized with sodium hydroxide before use.

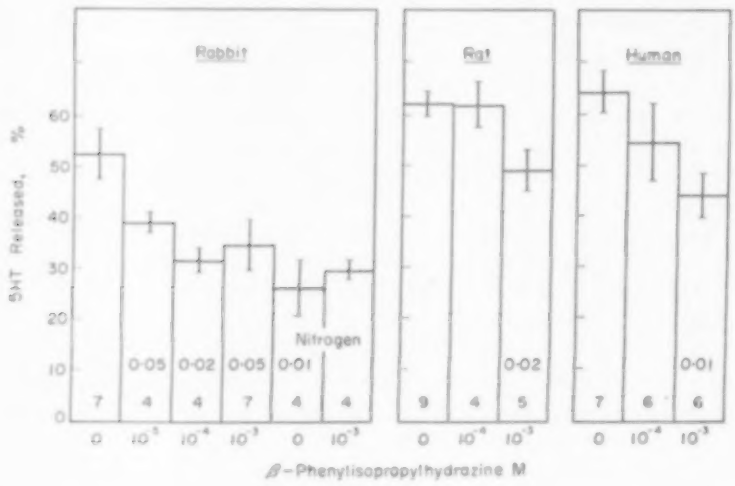

FIG. 1. Effect of β-phenylisopropylhydrazine on the amount of 5-hydroxytryptamine (5HT) in per cent (± s.e.m.) released from rabbit's blood platelets during 3-hr incubation of platelet-rich plasma with 20μg/ml of tetrabenazine. The incubations were done in air unless otherwise indicated. The significance of the differences between the means of the β-phenylhydrazine and corresponding control group are given only when $P<0{\cdot}05$. P is less than the over value in columns. The control group in nitrogen is compared to the control group in air. The lower value in columns indicates the number of experiments.

When PIH significantly increased the platelet 5HT (see Table 1) there was generally a roughly proportional increase in the number of platelets per ml of plasma, although this was not a consistent finding. The inhibition of tetrabenazine-induced 5HT decrease cannot be explained by higher numbers of platelets in the samples containing PIH in addition to tetrabenazine. Also, the low 5HT values obtained after incubation with high concentrations of the hydrazine derivatives were not due to the destruction of platelets.

DISCUSSION

In suitable concentrations MAO inhibitor β-phenyl*iso*propylhydrazine (PIH) inhibits the slight decrease in 5HT of the platelets (when estimated per ml of platelet-rich plasma), which normally occurs during incubation of platelet-rich plasma *in vitro*. The results indicate that fewer of the platelets are destroyed when incubated with PIH, but the reason, or an additional reason, may be MAO inhibition through mechanisms as discussed below in connection of tetrabenazine experiments. The role of MAO inhibition is emphasized by the findings that incubation in nitrogen instead of air had a similar effect to PIH, and a hydrazine lacking MAO inhibitory action, isoniazid,[13] had no effect.

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In concentrations of 10^{-3}M or higher, PIH itself lowered the 5HT content of platelets. At least on rat's platelets another MAO inhibitor, iproniazid, had similar effect. This action, however, was not bound to MAO inhibition, because isoniazid was more effective than iproniazid. The concentrations necessary for this release *in vitro* are beyond the therapeutic *in vivo* levels. The possibility of this action, however, has to be kept in mind at least while using relatively high doses of hydrazine derivatives in animal tests.

In addition to iproniazid, previously shown to inhibit reserpine induced depletion of 5HT from the platelets[6], PIH in various concentrations has inhibitory action on the tetrabenazine-induced 5HT release in platelets. PIH had an inhibitory effect in rabbit's platelets also in low concentrations which were ineffective in the platelets of the two other species. At least intact platelets of rabbits destroy their own 5HT released by tetrabenazine while platelets of man inactivate about one-third of their 5HT and those of rats less than 10 per cent of the amine released by this treatment.[8] The destruction of 5HT by platelets is inhibited if PIH is present in the medium, or if the incubation is carried out in an atmosphere of nitrogen.[8] Previous results also indicate that other MAO inhibitors inhibit this inactivation.[3, 14] PIH did not further increase the inhibition of tetrabenazine action produced by nitrogen. The inhibition mechanisms of PIH and nitrogen are probably similar, and involve the inhibition of 5HT oxidation.

In many respects the platelets are autonomous living cells with measurable metabolic functions.[15, 16] It is also probable that 5HT in the platelets is localized in subcellular particles[17] from which it is released by tetrabenazine to the cytoplasma. Therefore the mechanism for the inhibition produced by MAO inhibition for the 5HT depletion could be the same in platelets as in other tissues. Because the platelets have no 5-hydroxytryptophan decarboxylase activity[18] the picture is here less complex.

Unlike 5HT itself, its breakdown products can probably easily penetrate cell membranes. If MAO and 5HT are stored in different granules, as is suggested by studies on mastocytoma cells,[19] the amine has to penetrate the intracellular membrane before being metabolized, even when no MAO inhibitor is present. However, MAO inhibitor, with or without 5HT releasing agent, increases the free 5HT in the cytoplasm and this may, through the operation of the principle of mass action, prevent 5HT from coming out from the storage particle. The depletion of 5HT from the platelet could also be inhibited by the difficulty the free amine has in penetrating the cell membrane. Histological findings on intestinal mucosa[20] as well as those obtained by centrifuge from the brain homogenates[21] have indicated that iproniazid prevents to some degree the decrease of granular 5HT produced by reserpine. It is quite possible, however, that MAO inhibitors do not inhibit the fundamental action of substances like reserpine in preventing the cells from storing their 5HT normally, i.e. producing the change from bound to free amine as emphasized by Brodie *et al.*[22]

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THE INTRACELLULAR LOCALIZATION OF CARBONIC ANHYDRASE AND A CARBONIC ANHYDRASE INHIBITOR IN THE BRAINS OF MICE

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Abstract—Homogenates prepared from the perfused brains of mice were differentially centrifuged in 0·25 M sucrose. On the basis of activity per mg of nitrogen, only the supernatant showed a concentration of carbonic anhydrase activity greater than that of the original homogenate (about two- to three-fold). No concentration of activity was found in the particulate fraction, in any of its components studied, or in the washes of the various residues. Partition of per cent activity varied with the procedure used, but approximately two-thirds of the total activity of the original homogenate were in the supernatant fraction; the total particulate matter accounted for about one-third. No differences of any consequence were found amongst whole brain, cortex, and brain with the cortex removed. The data are believed to demonstrate the intracellular localization of carbonic anhydrase in the soluble fraction of cells; the activity of particulate matter is considered to be the result of contamination.

The inhibitor of carbonic anhydrase found in the brains of mice following the intravenous administration of methazolamide was almost entirely localized in the supernatant fractions; an intercellular localization in the soluble fraction is also postulated.

The localization of both carbonic anhydrase and carbonic anhydrase inhibitor in the soluble fraction of homogenates of the brains of mice does not constitute evidence of the inhibition of the enzyme in the brain *in vivo*. However, the concentrations of inhibitor found in the whole homogenates 15 min and 2 hr after the administration of doses of methazolamide at the level of the ED_{95} for anticonvulsant effect were theoretically capable of causing maximum inhibition of carbonic anhydrase in the brains of mice. Anticonvulsant action and inhibition of carbonic anhydrase are probably related.

INTRODUCTION

CONSIDERABLE circumstantial evidence has been published in support of the belief that the anticonvulsant action of inhibitors of carbonic anhydrase is attributable to inhibition of this enzyme in the brain.[1, 2] No direct proof of the inhibition of carbonic anhydrase in brain *in vivo* has been presented. Furthermore, since the times of the maximum concentration of inhibitor in the brain and the maximum anticonvulsant action are not coincident,[2] anticonvulsant action independent of carbonic anhydrase remains a possibility.

The present state of the art makes a direct demonstration of the degree of activity of carbonic anhydrase in brain *in vivo* following the administration of inhibitors extremely difficult, if not impossible. An indirect approach to the problem would be a distribution study with labeled inhibitor in conjunction with a histochemical method for the localization of carbonic anhydrase. Unfortunately, a reliable histochemical method for the detection of carbonic anhydrase is not available.[3] A crude but possibly

useful approach would be the differential centrifugation of brain homogenates. Location of the enzyme and an inhibitor in separate and discrete fractions would suggest that the anticonvulsant action is a pharmacodynamic effect independent of any action on carbonic anhydrase. It would also be of interest to calculate the possible degrees of inhibition of the enzyme in brain at various times following the administration of amounts of methazolamide causing a maximum anticonvulsant effect. The results of such a study are presented in this report.

METHODS

The mice used were male Swiss albino, from 6 to 9 weeks of age and weighing from 20 to 30 g. All reagents and solutions used in assay, perfusion and homogenization were prepared with double (glass)-distilled water. Except where indicated, perfused brains from mice anesthetized with sodium pentobarbital were used in the study of enzyme localization; unanesthetized mice sacrificed by cervical fracture furnished brains for the study of inhibitor distribution. These were rinsed in saline and blotted free of blood. Perfusion of the brain was carried out by placing small artery clamps on the abdominal aorta and on the inferior vena cava, then inserting a 20-gauge hypodermic needle into the left ventricle. Except where indicated, the perfusion fluid was 0·85 per cent saline. Immediately after perfusion had started, the right atrium was snipped. Perfusion was continued until no discoloration due to blood was observed on a piece of filter paper; usually a period of from 3 to 5 min was required. All brains, perfused and not perfused, were blotted dry and weighed on a torsion balance to the nearest milligram. Experiments showed no important differences in the localization of carbonic anhydrase in homogenates of perfused brains and of brains that had not been perfused.

Ten per cent homogenates (w/v) were prepared in 0·25 M sucrose by means of a glass homogenizer fitted with a Teflon®* pestle (A. H. Thomas Co., No. B-3235, clearance from 0·005 to 0·007 in., 220 rev/min, approximately ten strokes per min for from 3 to 8 min). When polyvinylpyrrolidone was used, the concentration in the sucrose was 7·3 per cent. Homogenization was carried out in an ice-bath. This precaution was omitted in the determination of inhibitor concentration. Lusteroid tubes were used throughout the centrifugation procedure. All residues, supernatant fractions and washes were made up to 10 ml with the suspending medium and stored at 3° until assayed. Equipment (tubes, etc). and suspending media were refrigerated at the same temperature. Assays for enzyme activity and inhibitor were carried out within 24 hr. Under these conditions, storage for 1 week had no effect on enzyme activity or concentration of inhibitor. Homogenates and the first and second residues were examined for intact cells and erythrocytes; counts of the latter were made when possible.

Localization of carbonic anhydrase

Of the 10% homogenate, 12 ml were differentially centrifuged in a Spinco model "L" ultracentrifuge (no. 40 rotor) at 5000 **g** for 10 min, 12,000 **g** for 15 min, and 45,000 **g** for 60 min; calculations of centrifugal force were made from the midpoint of the tube. These centrifugal forces were chosen arbitrarily since morphological characterization of the various fractions was not our main concern.

* Registered trademark of the E. I. duPont de Nemours and Company (Inc.).

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The first two residues were washed twice with the suspending medium. The final residue was washed once. In one experiment, the first centrifugation was 1000 **g** for 10 min. The effect of single high-speed centrifugation was also investigated.

An homogenate prepared in the usual way from the brains that had not been perfused was used to study the localization of enzyme following the addition of hemolysed mouse blood. It was divided into three 20-ml aliquots. One received an amount of hemolysed mouse blood containing about 150–160 units of carbonic anhydrase activity. The second sample was heated at 100° for 5 min. After cooling, the identical amount of hemolysed mouse blood was added. The third sample received an equal volume of the same hemolysed mouse blood which had been heated to 100° for 5 min and allowed to cool; all three samples were agitated by hand over a period of 5 min. Differential centrifugation was carried out in the manner previously described.

Localization of inhibitor

Methazolamide* was solubilized by the addition of one equivalent of sodium hydroxide per mole of compound. Fifty milligrams per kilogram in terms of the free acid, were given intravenously to mice; this is the ED_{95} for anticonvulsant effect in this species. Fifteen and 120 min later, the mice were sacrificed by cervical fracture. Homogenates were prepared as previously described. On the basis of the data obtained on the localization of carbonic anhydrase, 12-ml aliquots of these homogenates were partitioned into particulate and supernatant fractions by centrifugation at 45,000 **g** for 60 min. The residues were resuspended and recentrifuged under the same conditions. Homogenates prepared from the brains of untreated mice were centrifuged in the same manner; the particulate matter, wash, and supernatant fractions were heated at 100° for 5 min to furnish appropriate blanks.

Analytical methods

Enzyme activity and concentration of inhibitor were measured essentially in the manner described by Maren.[4] Three millilitres of phenol red were used instead of five. Control experiments showed that the assay of homogenates and fractions thereof in distilled water resulted in a loss of enzyme activity; there was also a spurious increase in the concentration of inhibitor. Consequently, the assays were carried out in such manner that the reaction volume of 7 ml contained amounts of sucrose or sucrose plus polyvinylpyrrolidone equivalent to 0·5 ml of the suspending medium.

The assay of enzyme activity was conducted as follows: aliquots of homogenates, residues, washes and supernatant fraction were heated at 100° for 5 min to furnish blanks in volumes of 0·1 ml to 0·5 ml for the construction of reference curves. Using arithmetic co-ordinates, the reciprocal of the uncatalysed reaction time in the presence of a known volume of the blank and the reciprocal of one-half this reaction time (equivalent to one unit) were used to plot a theoretical reference curve covering from 0 to 2 units of enzyme activity. A selected volume of the unknown, usually 0·1–0·2 ml, was then assayed. If the reciprocal of the reaction time was within the limits of the reference curve, two additional measurements were made. To be considered valid, these three reaction times could vary no more than 1 sec. The mean was used for the determination of enzyme units; the error was estimated to be $\pm$ 2 per cent. By definition, one unit of carbonic anhydrase activity is the amount necessary to reduce the uncatalysed reaction time by one-half in 7 ml of solution at 0.4°.

* The trademark of the American Cyanamid Company for methazolamide is Neptazane®.

In the measurement of the concentration of inhibitor, the reaction time of the unknown was adjusted by the reaction time of the same volume of appropriate blank. All measurements were made in triplicate and, to be considered valid, could vary no more than 1 sec; the error was calculated to be $\pm$ 3 per cent.

Nitrogen was determined by the direct nesslerization procedure of Hoffman and Osgood,[5] modified by the use of 30 per cent peroxide to reduce caramelization. All measurements were made in duplicate with a Beckman model "B" spectrophotometer; the error was $\pm$ 5 per cent.

Relative concentration of enzyme and S/P ratio were calculated as:

$$\frac{\text{units/mg N fraction or supernatant } (S)}{\text{units/mg N homogenate or total particulate matter } (P)}$$

Units of enzyme activity and percentages were rounded off to the nearest whole number; units of activity per mg of nitrogen were calculated to the first decimal place.

Calculation of enzyme inhibition

The theoretically possible degrees of enzyme inhibition in brain 15 min and 2 hr after the administration of doses of methazolamide at the level of the ED_{95} for anticonvulsant effect were calculated from the means of the number of enzyme units and inhibitor contents of the original homogenates given in Tables 1 and 3. From the data published by Maren *et al.*,[6] one enzyme unit in the carbonate buffer system was calculated to be $1{\cdot}68 \times 10^{-11}$ moles of carbonic anhydrase. The reaction conditions used in the present study and those of Maren and co-workers did not differ in important details. The equilibrium expression used was

$$K_I = \frac{[(E)-(EI)][(I)-(EI)]}{(EI)}$$

where (E) and (I) are the initial molar concentrations of free enzyme and inhibitor in intracellular water. The latter was calculated considering total water to be 78 per cent of the weight of the brains of mice.[7] On the basis of the estimate of extracellular space in brain made by Davson and Spaziani,[8] 85 per cent of the total water was considered to be intracellular. Acceptance of the existence of an extracellular space would introduce only a minor error into the calculations. In the above expression, $(I) - (EI)$ was not considered to be equivalent to (I). The value used for the equilibrium constant, K_I, was $0{\cdot}9 \times 10^{-8}$ M. This is the figure given by Maren *et al.*[6] for methazolamide in the carbonate buffer system. The degree of enzyme inhibition was calculated in per cent as

$$\frac{(EI)}{(E)} \times 100$$

RESULTS

1. *Localization of carbonic anhydrase activity in homogenates of whole brain*

Table 1 gives the means and the ranges of the data of three experiments in which the first centrifugation was carried out at 5000 **g** for 10 min. Thirty per cent of the enzyme activity and 52 per cent of the total nitrogen were found in the particulate matter, principally in the first residue. Relatively little enzyme activity and nitrogen were found in the 12,000 **g** and the 45,000 **g** fractions. Within the particulate matter, nitrogen content and enzyme activity appeared to be well correlated. The

supernatant accounted for 19 per cent of the nitrogen and 45 per cent of the total carbonic anhydrase activity of the original homogenate. The enzyme activity and nitrogen content of the combined washes were 20 and 28 per cent. The inclusion of 7·3 per cent of polyvinylpyrrolidone in the suspending medium had no action on the distribution of activity.

TABLE 1. THE LOCALIZATION OF CARBONIC ANHYDRASE FOLLOWING DIFFERENTIAL CENTRIFUGATION OF HOMOGENATES OF THE PERFUSED BRAINS OF MICE

Fraction	Activity			Nitrogen (N)		Relative Concentration
	Units	%	Units/ mg N	mg	%	
Homogenate	119 (114–126)	100 –	4·6 (4·2–5·0)	25·8 (25·2–27·0)	100 –	1·0
5000 g, 10 min	28 (21–35)	23 (18–28)	2·8 (2·1–3·3)	10·2 (9·8–10·5)	39 (36–42)	0·6 (0·4–0·7)
12,000 g, 15 min	5 (4–7)	4 (3–6)	3·1 (2·3–3·7)	1·8 (1·3–2·0)	7 (5–8)	0·7 (0·6–0·7)
45,000 g, 60 min	2 (2–2)	2 (2–2)	1·6 (1·2–1·8)	1·5 (1·3–1·7)	6 (5–6)	0·4 (0·3–0·4)
*Total Particulate (*P*)	36 (27–45)	30 (23–36)	2·6 (2·1–3·2)	13·4 (12·9–13·8)	52 (50–55)	0·5 (0·4–0·6)
Supernatant fraction (*S*)	54 (50–57)	45 (40–50)	11·2 (11·1–11·3)	4·8 (4·5–5·1)	19 (18–19)	2·4 (2·2–2·7)
Washes (*W*)	24 (21–28)	20 (18–24)	3·3 (2·8–3·9)	7·3 (7·2–7·4)	28 (27–29)	0·7 (0·6–0·8)
S + *W*	78 (72–83)	65 (57–70)	6·4 (6·1–6·8)	12·1 (11·8–12·5)	47 (46–47)	1·4 (1·3–1·5)
Recovery	113 (110–117)	95 (93–99)	–	25·5 (25·0–26·0)	99 (96–102)	–

Data in the table are the means and the highest and lowest values (in parentheses) of three experiments. Ten per cent homogenates in 0·25 M sucrose. Discrepancies in balance are due to rounding off the original data (see Methods).

* *S*/*P* ratio = 3·6.

$$\text{Relative concentration and } S/P \text{ ratio} = \frac{\text{units/mg N fraction or supernatant fraction } (S)}{\text{units/mg N homogenate or total particulate matter } (P).}$$

The relative concentration of activity in the supernatant fraction was more than two-fold that of the original homogenate and about four times that of the total particulate matter (*S*/*P* ratio); in the particulate matter and in each of its components it was less than that of the original homogenate. The individual washes from each residue showed no concentration of activity and consequently were pooled as shown in the table. In general, the second wash from a residue contained about half the enzyme activity of the first wash on a percent basis; nitrogen content was also diminished.

The substitution of 1000 **g** for 5000 **g** for 10 min did not cause any change in the relative concentration of enzyme activity. Single high-speed centrifugation at 45,000 **g** for 60 min resulted in an equal partition of per cent activity between the particulate matter and the supernatant fraction. The nitrogen content of the particulate matter was increased to 79 per cent, while that of the supernatant fraction was relatively unchanged (21 per cent). Compared with the multiple fractionation procedure, there were no changes in relative concentration in terms of the original homogenate or the particular matter (*S*/*P* ratio). The aliquot of the same homogenate centrifuged at 100,000 **g** for 60 min showed 40 per cent of the enzyme

activity and 82 per cent of the nitrogen in the particulate fraction. The supernatant fraction now contained 60 per cent of the activity; the nitrogen content, 18 per cent, again was little changed. The relative concentration of activity in the supernatant fraction was increased to some degree compared to the single 45,000 g centrifugation or the multiple fractionation procedure. The *S/P* ratio of 6·8 was a large increase over previous ratios.

When fractionated in the manner described in Table 1, there were no differences of any consequence amongst whole brain, cortex, and whole brain with the cortex removed.

TABLE 2. THE LOCALIZATION OF CARBONIC ANHYDRASE FOLLOWING THE ADDITION OF THE ENZYME TO UNTREATED AND BOILED HOMOGENATES OF THE BRAINS OF MICE

Condition	Fraction	Activity (units)	Activity (%)	Activity (units/mgN)	Nitrogen (N) (mg)	Nitrogen (N) (%)	Relative concentration
Untreated plus enzyme (A)	Homogenate	295	100	12·8	23·0	100	1·0
	5000 g, 10 min	112	38	16·6	6·8	30	1·3
	12,000 g, 15 min	4	1	2·7	1·5	6	0·2
	45,000 g, 60 min	3	1	2·2	1·6	7	0·2
	*Total particulate (*P*)	120	41	20·0	9·8	43	1·6
	Supernatant fraction (*S*)	128	43	22·0	5·8	25	1·7
	Washes (*W*)	43	15	5·8	7·3	32	0·5
	S + *W*	171	58	13·0	13·1	57	1·0
	Recovery	291	99	—	22·9	100	—
Boiled plus enzyme (B)	Homogenate	166	100	7·6	21·8	100	1·0
	5000 g, 10 min	85	51	13·9	6·1	28	1·8
	12,000 g, 15 min	1	1	0·6	1·8	8	0·1
	†45,000 g, 10 min	2	1	0·6	3·3	15	0·1
	Total particulate (*P*)	88	53	7·9	11·2	52	1·0
	Supernatant fraction (*S*)	67	40	9·3	7·2	33	1·2
	Washes (*W*)	12	7	3·4	3·6	16	0·4
	S + *W*	79	47	7·3	10·8	49	1·0
	Recovery	167	100	—	22·0	101	—
Untreated plus boiled blood (C)	Homogenate	149	100	6·8	22·0	100	1·0
	5000 g, 10 min	46	31	6·1	7·5	34	0·9
	12,000 g, 15 min	6	4	4·1	1·5	7	0·6
	45,000 g, 60 min	4	3	2·5	1·5	7	0·4
	Total particulate (*P*)	56	38	5·3	10·5	48	0·8
	Supernatant fraction (*S*)	62	42	12·2	5·0	23	1·8
	Washes (*W*)	35	24	5·2	6·7	30	0·8
	S + *W*	97	66	8·3	11·7	53	1·2
	Recovery	152	102	—	22·2	101	—
	Original homogenate	145	100	5·9	24·4	100	—

The brains were not perfused. A 10% homogenate in 0·25 M sucrose was divided into three 20-ml aliquots. A and B received 150–160 units of carbonic anhydrase activity (hemolyzed mouse blood); B received the enzyme after being heated at 100°C for 5 min; C received the same amount of hemolyzed mouse blood heated at100°C for 5 min.

Relative concentration and S/P ratio calculated as shown in Table 1.

* *S/P* ratio = 1·1.

† *S/P* ratio = 1·2.

‡ *S/P* ratio = 2·3.

2. *Localization of activity following the addition of enzyme to brain homogenate*

The particulate matter of both untreated and boiled homogenates showed a great capacity to carry down carbonic anhydrase activity upon centrifugation; these data are summarized in Table 2. The added activity in each instance was about equally

partitioned between the supernatant fraction and the total particulate matter. The first residue (5000 g, 10) was the sole carrier. Only the boiled homogenate differed importantly from the untreated aliquot receiving only boiled blood. The first residue accounted for about 51 per cent of the total activity and the combined washes for only 7 per cent. The distribution of nitrogen differed to some degree from the established pattern. The addition of enzyme to untreated homogenate resulted in a slight increase in the relative concentration of activity in the first residue, with no change in that of the supernatant fraction. An increase was found in the particulate matter; there was no difference between it and the supernatant fraction ($S/P = 1{\cdot}1$). The relative concentration of activity in the 5000 g residue of the boiled homogenate was significantly increased, being nearly twice that of the whole homogenate. The concentrations of activity in the latter and in the total particulate and supernatant fraction were practically identical.

Compared with the aliquot receiving boiled blood, the concentrations of activity in the 12,000 g and the 45,000 g residues of untreated and boiled homogenates were reduced to some degree.

TABLE 3. THE LOCALIZATION OF CARBONIC ANHYDRASE INHIBITOR FOLLOWING CENTRIFUGATION OF HOMOGENATES OF THE BRAINS OF MICE

Fraction	A		B	
	(μg)	(%)	(μg)	(%)
Homogenate	11·1 (9·6, 12·6)	100	5·6 (6·0, 5·3)	100
45,000 g, 60 min	1·0 (1·2, 0·8)	9 (12, 6)	1·1 (1·2, 1·0)	20 (20, 19)
Supernatant fraction (S)	9·4 (7·3, 11·4)	83 (76, 90)	3·8 (4·0, 3·6)	68 (67, 68)
Wash (W)	1·2 (0·9, 1·4)	10 (9, 11)	0·6 (0·7, 0·5)	11 (13, 9)
$S + W$	10·5 (8·2, 12·8)	93 (85, 101)	4·4 (4·7, 4·1)	78 (78, 77)
Recovery	11·5 (9·5, 13·6)	104 (99, 108)	5·5 (6·0, 5·0)	97 (100, 94)

Ten per cent homogenates in 0·25 M sucrose; the brains were not perfused. A was from mice sacrificed at 15 min and B from mice sacrificed at 2 hr after the intravenous administration of 50 mg of methazolamide per kg. The data are the means and individual values obtained in two separate experiments. With the exception of the original homogenate (12 ml), the various fractions and washes made up to 10 ml with 0·25 M sucrose; conversion of the data to μg/ml can be readily made.

3. *Localization of carbonic anhydrase inhibitor*

Fifteen minutes after the intravenous administration of methazolamide, 83 per cent of the total inhibitor activity was found in the supernatant; the particulate matter accounted for 9 per cent (Table 3). The supernatant fraction and the wash accounted for over 90 per cent of the total amount of inhibitor present. Essentially similar results were obtained with an homogenate prepared from perfused brains. The concentration of inhibitor in the particulate matter is not due to residual blood. The inclusion of 7·3 per cent of polyvinylpyrrolidone in the suspending medium had

no effect. Two hours after administration, 68 per cent of the inhibitor present was found in the supernatant fraction; the particulate matter contained 20 per cent. The absolute amount of inhibitor in this fraction did not change with time.

When methazolamide (sodium salt) was added to brain homogenate, the amount found in the particulate matter was the same as that found after *in vivo* administration.

Perfusion of the brains of mice with normal saline up to 15 min had no action on the concentration of inhibitor. In two experiments, sucrose perfusion for a period of 15 min caused reductions of 19 and 28 per cent; in one experiment it was without effect. All perfusions were equally effective in reducing the erythrocyte count.

4. *The calculated degrees of the inhibition of carbonic anhydrase in brain*

On the basis of the procedure given in the section on Methods, the molar concentration of carbonic anhydrase in the brains of mice was calculated to be $2{\cdot}534 \times 10^{-6}$ M. (*EI*) was $2{\cdot}534 \times 10^{-6}$ M at both 15 min and 2 hr after the intravenous administration of amounts of methazolamide estimated to have an anticonvulsant effect in 95 per cent of a population of mice. Consequently, maximal inhibition (100 per cent) of the enzyme in brain appears to be possible at these times, provided that all of the enzyme and all of the inhibitor are in immediate contact.

DISCUSSION

The heterogeneity of the cell population of the brain restricts the usefulness of the technique of differential centrifugation. It is not known if the carbonic anhydrase in brain is located in certain specific cells or in all cells. Conceivably, the enzyme and the inhibitor could be located in different cells, but following centrifugation both could end up in the supernatant fraction of the whole homogenate. Conclusions drawn about the situation *in vivo* would be entirely spurious. A further difficulty with this technique is the likelihood of the removal of water-soluble substances from the particulate matter into the aqueous phase. The localization of an agent in the extracellular space would also be misinterpreted as location in the soluble fraction. However, in view of the rather unsettled state as to the existence of extracellular space in brain,[8, 9] there is not much to be gained in pursuing this possibility further.

The inhibitor found in brain has not been identified; however, there is no reason to believe that it is not methazolamide. At pH 7·3, the solubility of methazolamide in water is about 3·5 mg/ml; at pH 2·0 it is about 1 mg/ml. The free acid is relatively insoluble. Theoretically, all inhibitor present in brain could be leached out of the particulate matter into the suspending medium. However, solubility alone does not dictate distribution in biological systems. Maren *et al.*[10] found that methazolamide was not readily washed out of human and canine red blood cells, even though the solubility of methazolamide greatly exceeds the amounts of inhibitor present. The unionized moiety most probably is the form that enters into the brain; the p*Ka* of methazolamide is 7·4. Consequently, at physiological pH, about one-half of the amount in brain could exist in the ionized state. If methazolamide were actually located in the particulate matter, a considerable amount, perhaps one-half, might not be able to cross boundaries at the pH of the suspending medium (6·5). The binding of agents of this type by tissue components is an additional factor to be taken into consideration (Maren *et al.*[10]). The above considerations support the belief that the

presence of nearly all of the inhibitor in the supernatant fraction represents intracellular localization in the soluble fraction; its presence there does not appear to be an artifact due to the leaching of methazolamide out of the particulate matter into the aqueous phase. If an extracellular space exists in the brain, methazolamide does not appear to be predominantly localized there. Perfusion with saline or 0·25 M sucrose had no significant action on the concentration of inhibitor. The small amount of inhibitor found in the particulate matter most probably represents contamination. The constancy of the amount found in the particulate matter at different times following intravenous administration further suggests that its presence there is not fact, but artifact. The distribution of inhibitor following the addition *in vitro* of the water-soluble sodium salt of methazolamide to brain homogenate may not be pertinent. However, it does show that the particulate matter can carry down an amount of inhibitor of the same order of magnitude as found when methazolamide is given intravenously.

The carbonic anhydrase activity in the supernatant fraction appears to represent intracellular localization in the soluble fraction. The evidence is as follows: only in the supernatant fraction is the concentration of carbonic anhydrase activity (in terms of units per mg of nitrogen) significantly greater than that of the original homogenate. The activity in the supernatant fraction does not appear to be due to the leaching or the leaking of the enzyme out of the particulate matter; the concentration of activity in the washes of the various residues was always less than that of the original homogenate or of the supernatant fraction. Furthermore, with reference to the residue washed, the second wash contained a much smaller per cent of the residual activity than did the first. The inclusion of polyvinylpyrrolidone, which is considered to stabilize biochemical and morphological characteristics,[11, 12] was without effect.

The carbonic anhydrase activity in the particulate matter is the result of its great ability to carry down the enzyme, as shown by: (1) the absence of any concentration of activity in the total particulate matter or any of its fractions studied, relative to the original homogenate; (2) the nitrogen content of the individual residues was closely correlated with enzyme activity (within the particulate fraction, the partition of carbonic anhydrase on a per cent basis was directly related to the amount of material present as measured by nitrogen content); (3) the activity of the particulate matter was not independent of the force of centrifugation, i.e. 100,000 **g** for 60 min reduced the per cent of the total activity in the particulate matter; (4) erythrocytes and other unbroken cells that could contribute a considerable amount of carbonic anhydrase activity were only occasionally seen in the 5000 **g** residue, and never were found in the 12,000 **g** fraction; and (5) the direct demonstration of the great capacity of the particulate matter to carry down enzyme following addition *in vitro*.

Datta and Shepard[12] have also concluded that carbonic anhydrase is localized in the soluble fraction of the livers and the kidneys of rats. Data published recently by Karler and Woodbury[14] are claimed to show that the enzyme is located in both the soluble fraction and mitochondria of the kidneys and cerebral cortices of rats. Examination by the light microscope showed that the 5000 **g** residue of the present study was mostly nuclei, debris and occasionally an unbroken cell. The 12,000 **g** residue consisted of particles morphologically similar to mitochondria[15] and could be stained with Janus Green B. Functional studies as an aid to identification were not

carried out. This residue of the cerebral cortices of the brains of mice might correspond to what Karler and Woodbury consider to be the mitochondrial fraction of the cerebral cortices of rats. The activity was only 7 per cent of the total, in relative concentration was not materially different from the original homogenate, and is considered to be the result of contamination.

The technique of differential centrifugation has produced evidence, admittedly gross, that the carbonic anhydrase in the brains of mice and the inhibitor found there following the administration of methazolamide are very probably localized intracellularly in the soluble fraction. This, of course, does not constitute proof of the inhibition of the enzyme *in vivo* or even of location in the same cell. Theoretically, at both times studied, there would appear to be sufficient inhibitor present to cause a maximum inhibition of all the carbonic anhydrase in the brains of mice *if* all of the enzyme and all of the inhibitor were in intimate contact. These calculated degrees of enzyme inhibition should be received with considerable reservation and extrapolated to the situation *in vivo* with caution because of: (1) the uncertainty regarding the equilibrium constants (K_I) (for example, the K_I of acetazolamide determined by Keller *et al.*[16] in carbonate buffer is about one-sixth of that found by Maren *et al.*[6]; (2) the concentration of "free" inhibitor (I) may be seriously overestimated because of (a) the known binding of inhibitors by other tissue components,[10] and (b) a different anatomical distribution pattern of enzyme and inhibitor; (3) the demonstration by Keller *et al.*[16] that carbonic anhydrase itself apparently has an additional binding site besides the active centre (this second binding site has been neglected in the equilibrium expression); (4) the estimations of the degrees of enzyme inhibition have not been extended to 37 °C; and (5) pending the purification of carbonic anhydrase, the possibility of error in expressing the concentration of enzyme in molar terms should be kept in mind.

With all of its limitations, the results of this study cannot be considered conclusive. However, no evidence was found that the anticonvulsant action of this class of compounds is unrelated to the inhibition of carbonic anhydrase in brain.

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THE EFFECT OF EXPERIMENTALLY MODIFIED SLEEPING PERIODS ON BRAIN LEVELS OF BARBITAL*

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Abstract—Whole brain levels of barbital were determined in 158 albino rats. The drug levels in the brain were related to experimentally induced alterations in the latent and sleeping periods following barbital injection. The brain barbital levels were related to observed alterations in latent and sleeping periods. Significant changes in latent times and in periods of sedation occurred in the absence of substantial differences in the brain levels of barbital from those measured in control animals at equivalent times following the injection of barbital.

ALTERATIONS in the duration of barbiturate hypnosis have been induced experimentally by the use of a wide variety of pharmacological agents. As pointed out by Richards in his excellent review of the subject,[1] several mechanisms may be operative in the modification of barbiturate effects. The chemical makeup and metabolic fate of the barbiturates are factors, as well as the dosage and species employed. Many of the previous investigations concerned with the duration of the sedative effects of the barbiturates have not included data on brain levels of the drug, so that accurate criteria for analysis of possible modes of action have frequently not been available.

The purpose of this investigation was to study the effect of experimentally produced alterations in the duration of barbiturate-induced sedation on the level of the drug in brain tissue at the beginning and end of sedation. In order to obviate variability that might occur due to changes in barbiturate biotransformation, barbital, which is not metabolized and is excreted unchanged, was employed for the induction of sedation.

METHODS

Adult albino rats of the Denver strain were employed as experimental animals. Sedation was induced by the intraperitoneal injection of 200 mg of sodium barbital per kg. A latent period was measured from the time of injection to loss of the righting reflex. Sleeping periods were measured from the time of loss of righting reflex to the return of the same reflex. All animals were killed by decapitation and the entire brain was removed, weighed and immediately homogenized. Total brain barbital levels were determined for each animal by the method of Brodie,[2] in which the tissue is extracted with petroleum ether in acid solution. The drug is then transferred from

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the ether to an aqueous alkaline phosphate buffer and quantitated spectrophotometrically. Brain levels of barbital were expressed as micrograms per gram of brain tissue. Brain levels of barbital determined in a series of control groups of male rats at the onset of sleep, the end of the sleeping period, 80 min, 210 min and 300 min respectively, after injection with sodium barbital. The drug was administered intraperitoneally as a saline solution in a dose of 200 mg kg. A group of male animals were treated with reserpine, 100 μg per kg, injected subcutaneously. Twenty hours following the administration of reserpine, the animals were administered sodium barbital. In another group of male rats, 19·5 hr following treatment with the same dose of reserpine, lysergic acid diethylamide (LSD) was injected subcutaneously in a dose of 1 mg per kg, followed in 30 min by the injection of the barbital. One group of male rats was injected with sodium barbital dissolved in distilled water. Two groups of female animals were utilized. A control group was injected with sodium barbital in distilled water via the intraperitoneal route. A second group of females was subjected to bilateral ovariectomy and administered the same dose of barbital 9 days after operation. Brain levels of barbital were determined in all of the experimental groups at the end of the latent period and at the end of the sleeping period. The dose of barbital given intraperitoneally, was 200 mg per kg in all animals.

RESULTS

Latent periods and sleeping times observed for the various groups of animals are summarized in Table 1. Significant differences from control values were observed in the latent periods in animals treated with reserpine plus LSD, and, in the groups in which distilled water was employed as the vehicle for barbital. Administration of reserpine prior to barbital sedation resulted in greatly prolonged sleeping times. Injection of LSD after treatment with reserpine prior to induction of barbital hypnosis was followed by sleeping times greatly reduced from those seen in the reserpine-treated animals. These observations on the effect of reserpine, and of reserpine plus LSD, on sleeping times are in accord with results reported by Taeschler and Cerletti[3] for thiopental and Brodie *et al.*[4] for hexobarbital. The sleeping times of castrate females were generally longer than those for female controls, but due to considerable variability from animal to animal the difference was not statistically significant. No significant differences were noted between the latent periods or sleeping periods in male and female animals of the control groups, with distilled water as the vehicle for the barbital.

Brain levels of barbital at the beginning and at the end of sleep are presented in Table 2, and are related graphically to latent and sleeping periods in Fig. 1. Included in the data are brain levels of the drug in control animals at various intervals of time following barbital injection. In the group treated with reserpine prior to barbital injection, no significant difference occurred in the brain levels of barbital between these animals and the brain levels in control animals, at equivalent times following injection of barbital. From Fig. 1 it may be seen that the brain level of the drug in the animals treated with reserpine and LSD, as they awoke, was not greatly lower than the level in reserpine-injected, sleeping animals at the same point in time. Very small differences in brain levels of barbital were recorded between female control and castrate groups, and the male group given barbital in distilled water, although most of the castrate females slept for much longer periods. In the presence of significant

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TABLE 1. LATENT PERIODS AND SLEEPING PERIODS AFTER SODIUM BARBITAL INJECTION

	N	Dose (mg/kg)	Latent period (min)	s.e.	*N*	Sleeping period (min)	s.e.	*P*	
Controls, male barbital, saline	26	200	46·7	±1·86	30	122·4	±6·17		
Males, reserpine + barbital, saline	20	0·1 200	41·0	±1·60	10	227·0	±25·30	LP vs. controls SP idem	>0·01 <0·001
Males, reserpine + LSD + barbital, saline	21	0·1 1·0 200	37·0	±1·64	11	98·0	±11·10	LP vs. controls SP idem LP vs. reserpine SP idem	<0·001 >0·01 idem <0·001
Males, barbital in dist. H_2O	25	200	68·0	±3·10	14	149·0	±11·17	LP vs. controls SP idem	<0·001 >0·01
Controls, female barbital in dist. H_2O	19	200	73·0	±3·60	10	118·0	±13·00	LP vs. males, dist. H_2O SP idem	>0·01 idem
Females, ov'cty barbital in dist. H_2O	18	200	55·6	±2·59	9	160·0	±23·50	LP vs. female controls SP idem	<0·001 >0·01

LP latent period.
SP sleeping period.

TABLE 2. BRAIN LEVELS OF BARBITAL DURING AND AFTER SODIUM BARBITAL SEDATION

			Brain levels μg barbital/gm brain					
	N	Dose (mg/kg)	At end of latent period	s.e.	*N*	At end of sleeping period	s.e.	*P*
Controls, male barbital, saline	10	200	79·1 (a)	±2·54	14	83·1 (b)	±2·29	
Controls, male barbital, saline	11	200	Killed 80 min after injection			82·9 (c)	±2·74	
Controls, male barbital, saline	12	200	Killed 210 min after injection			82·0 (d)	±2·15	
Controls, male barbital, saline	9	200	Killed 300 min after injection			67·9 (e)	±1.18	
Males, reserpine + barbital, saline	10	0·1 200	70·8 (f)	±3·20	10	67·5	±2·70	LP vs. a >0·01 SP vs. e idem
Males, reserpine + LSD + barbital, saline	10	0·1 1·0 200	71·3	±1·80	11	74·2	±2·24	LP vs. f >0·01 SP vs. b <0·01
Males, barbital in dist. H_2O	10	200	83·2 (g)	±2·84	14	73·7 (h)	±2·19	LP vs. c >0·01 SP vs. d <0·01
Controls, female barbital, dist. H_2O	9	200	75·2	±1·75	10	73·5	±1·09	LP vs. g >0·01 SP vs. h idem
Female, ov'cty barbital, dist. H_2O	9	200	76·1	±1·40	9	70·8	±2·11	LP vs. female controls >0·01 SP idem idem

LP brain level at end of latent period.
SP brain level at end of sleeping period.

differences in latent periods which occurred in the various experimental groups, no equivalent differences were noted in the brain levels of barbital at the onset of sleep. The animals injected with reserpine awakened on an average of 260 min after the injection of barbital, with brain levels of barbital which were essentially the same as those of the control animals which had been awake on the average 100 min. A greater

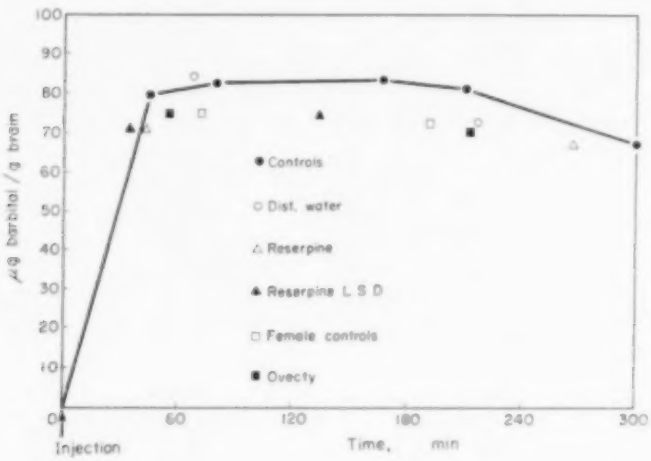

FIG. 1. Brain levels of barbital at the beginning and at the end of barbital sedation. Symbols between 0 time and 80 min on the time scale represent brain levels of barbital at the induction of sleep. Symbols to the right of the 120 min mark show the brain levels of barbital at the end of sleep. Solid line and circles indicate male control animal brain levels.

than 50 per cent reduction of the reserpine-induced prolonged sleeping period was achieved by LSD administration without the occurrence of any large difference in the brain level of barbital.

DISCUSSION

It is of interest to note that these data are in agreement with those of Giarman *et al.*[5] for thiopental, in that a critical level of barbital must be attained prior to induction of sleep. Those workers clearly showed that below a similarly critical level of thiopental, consciousness invariably returned. In the case of barbital, awakening can occur at any point along the gradually descending curve of brain barbital content. It should be noted that, in our experiments, a single standard dose of barbiturate was employed, and that no bio-transformation is involved with barbital.

The results of these experiments suggest that the brain tissue content of barbital is not the primary determinant of the duration of sedation, once sleep is induced. Since alterations in metabolic rate and degradation are not important factors with this particular drug, other mechanisms must be considered. Changes in the rate of excretion could affect the duration of sedation, but elevated brain levels of the drug would be expected. It is possible that the brain cells may be conditioned to react quantitatively in a different manner to equivalent amounts of barbital by virtue of

relative estrogen lack, or of altered quantities of brain amines, initiated by the neuropharmacological actions of drugs such as reserpine or LSD.

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SHORT COMMUNICATIONS

Transport of vitamin B_{12} from blood to cerebrospinal fluid

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PRELIMINARY investigations[1] have shown that there is no simple correlation between the concentration of vitamin B_{12} in serum and that in the cerebrospinal fluid (CSF) in human beings. These observations led to investigations of the transport of vitamin B_{12} from blood to CSF. Apparently no earlier publications have appeared on this subject.

Albino rabbits of homogeneous descent and breed were used for the experiments. The animals were anaesthetised with urethan (1·5 g/kg body weight). During the surgical operation the anaesthesia was supplemented with ether. Blood specimens were withdrawn from the femoral vein after bilateral ligation of the renal arteries and veins.

Cyanocobalamin (vitamin B_{12}) (25 μg/kg body weight) was then injected into an ear vein. Thirty minutes later another blood specimen was withdrawn. The experiment was concluded by exsanguination of the animal during which a further blood specimen was taken. Finally the maximum amount of CSF (0·5–2·0 ml) was withdrawn from the cisterne magna with a cannula.

Vitamin B_{12} in serum and CSF was assayed microbiologically by means of the *Euglena gracilis, z-strain* method[2] using a standard solution of cyanocobalamin containing 5 $\mu\mu$g/ml. The standard deviation of the assay was ± 18·0 per cent over the whole range of measurement. In serum both the total and the non-dialysable vitamin B_{12} were assayed, the former after heating at 100° for 15 min, the latter after dialysis against two changes of 10 vols of 0·0005 per cent aqueous KCN for 16 hr.

A control group of animals of the same descent and breed as the treated animals were submitted to a similar procedure except that vitamin B_{12} was not injected.

TABLE 1. THE TRANSFER OF VITAMIN B_{12} FROM BLOOD TO CSF, IN RABBITS (EACH LINE REFERS TO A SEPARATE ANIMAL).

Duration of experiment (min)	Total concentration of B_{12} in serum before injection (C_{serum}) (mμg/ml)	Total concentration of B_{12} in CSF (C_{CSF}) (mμg/ml) after injection	$\frac{C_{CSF}}{C_{serum}} \times 100$	Concentration of dialysable B_{12} in serum* after injection ($C_{serum-dialys.}$) (mμg/ml)	$\frac{C_{CSF}}{C_{serum-dialys.}} \times 100$	Total concentration of B_{12} in serum, (mμg/ml)	Total concentration of B_{12} in CSF, (mμg/ml)	$\frac{C_{CSF}}{C_{serum}} \times 100$
30	5·4	0·396	7·7	55·0	0·72	3·0	0·12	4·00
60	8·7	1·78	20·4	91·5	1·95	2·4	0·10	4·16
90	11·0	1·71	15·6	125·1	1·37	2·4	0·08	3·34
90	1·5	0·378	25·2	128·3	0·29	15·0	0·25	1·67
135	3·9	0·279	7·4	9·8	2·93	1·4	0·06	4·30
180	10·2	1·13	11·0	91·5	1·23	4·6	0·10	2·18
180	7·2	0·810	11·2	72·8	1·11	2·8	0·08	2·86
180	3·6	0,347	9·7	49·5	0·70	7·0	0·13	1·86
180	5·1	0·662	13·0	54·8	1·20	3·4	0·14	4·10
						1·6	0·06	3·75
	M: 6·59	M: 0·902	M: 14·2		M: 1·07	M: 4·36	M: 0·112	M: 3·22
	S.D.: ± 3·29					S.D.: ± 4·05	S.D.: ± 0·056	S.D.: ± 1·01

* Estimated as the difference between total and non-dialysable B_{12}

A comparison of the results in the table for treated and control animals shows that the concentration of vitamin B_{12} in the CSF is significantly higher in the treated group than in the controls. Furthermore considerable amounts of vitamin B_{12} have been transported to the CSF as shown by the fact that the ratio of the vitamin B_{12} level in CSF to the pre-injection serum level is 4 times higher in the treated group than in the control group.

The ratio CSF-B_{12}/dialysable-B_{12} in serum during the experiments is explicitly lower than the ratio CSF-B_{12}/serum-B_{12} in the control group.

Although only the concentration of dialysable vitamin B_{12} is brought into calculations in the treated group,[3] it is evident that equality is not reached since the ratio has not been as high as in the normal controls. (It has not been possible to demonstrate dialysable vitamin B_{12} in the normal rabbit-serum.)

The ratios show considerable variation but without any significant correlation to time as would have been expected. This may be due to variations in the age of the rabbits, since the blood-brain barrier is known to vary with age.[4]

From these experiments no conclusions can be drawn regarding the mechanism of the transport of vitamin B_{12} from blood to CSF, but the results demonstrate that vitamin B_{12} is transported from blood to CSF with relative ease, since, as already mentioned, the concentration of vitamin B_{12} is raised in the CSF 30 min after injection of the vitamin into the blood stream.

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5-Bromo-2′-deoxycytidine (BCDR)–I. Studies on metabolism *in vitro* and in mice

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HALOGENATED pyrimidine derivatives have been known for more than half a century, but investigations of their metabolic activity in mammalian systems have been conducted only recently. Although 5-iodo-2′-deoxyuridine (IUDR) markedly inhibited the growth of experimental neoplasms in mice,[1] the results obtained in man have been only moderately encouraging.[2] Whether this discrepancy may be explained by the more rapid catabolism of IUDR in man,[2, 3] as compared to the mouse,[4] is not yet clear. In both species there is rapid cleavage of IUDR to 5-iodouracil and dehalogenation to uracil.[3, 4]

Because halogenated derivatives of deoxyuridine can lower the threshold of mamalian cells to radiation-injury,[5, 6] the analogous derivatives of 2′-deoxycytidine have been investigated in the hope that these molecular species might have greater metabolic stability, a circumstance which could result in their more extensive incorporation into the acid-soluble nucleotide pools, as well as into deoxyribonucleic acid (DNA). The possible advantage of the use of the deoxycytidine derivatives as chemotherapeutic agents, because of their potentially greater resistance to metabolic degradation, has been discussed elsewhere.[7] Biochemical evidence suggests that the presence of the amino group, in lieu of oxygen in position 4 of the pyrimidine ring, should result in greater metabolic stability.[8, 9] These studies have shown that the enzyme, deoxycytidylate (dCMP) deaminase, present in rapidly growing mammalian tissues, e.g., neoplasms, embryonic tissues, regenerating liver, thymus, and bone marrow, is absent or is suppressed,[8, 9, 10] in most normal tissues. Thus, 5-bromo-2′-deoxycytidine (BCDR)[11] and 5-iodo-2′-deoxycytidine (ICDR)[12] might be expected to exhibit relatively selective action for tumour tissues, since many mouse, rat and human neoplasms are rich in dCMP deaminase. A previous note[13] has demonstrated that ^{3}H-BCDR is incorporated into the DNA of cells as 5-bromo-2′-deoxyuridine 5′-phosphate, replacing an equivalent amount of thymidylic acid, but it is not yet

certain whether phosphorylation precedes or follows deamination. However, the metabolic conversion of BCDR to deaminated derivatives has been observed both in a cell-free system and in intact neoplastic murine mast cells (P815Y).[12, 14]

The triphosphate of BCDR was first synthesized by Markham, as reported by Bessman *et al.*[15]; later, Chang and Welch[11] described an improved synthesis of BCDR, as well as the preparation of the ^{3}H-labeled compound. In an abstract by Cramer *et al.*[13] and in an oral report by Kriss *et al.*,[16] the latter describing the use of ^{82}Br-BCDR, BCDR was shown to be more stable metabolically in the mouse and rat than is 5-bromo-2′-deoxyuridine (BUDR).

The present report is concerned with studies of the metabolism of ^{3}H-BCDR.

Studies in vivo. Ten mice were placed individually in glass beakers and the urine was collected (for either a 4-hr or a 24-hr period) on filter paper protected by a wire screen. Following the administration to each mouse of a single injection of ^{3}H-BCDR (1 μmole; 6 × 10^5 cpm), 5 mice were sacrificed after 4 hr and the remainder after 24 hr. The filter paper was eluted with water in a Waring blendor and the eluate was filtered; this extract, adjusted to pH 10·5, was passed through a Dowex-1 formate column (200–400 mesh, 30 × 2·5 cm); the column was subjected to gradient elution with formic acid as was done previously in similar studies of IUDR.[4] Although the many compounds excreted have not yet been identified or quantitated, at least six major radioactive fractions were obtained in the 4-hr specimen of urine, and at least eight such fractions were present after 24 hr.

It has been reported that following the administration of either 6-azathymine,[17] IUDR,[4] or 5-bromouracil,[18] free uracil appears in the urine of mammalian species; these studies have shown that BCDR causes a similar result. The uracil excreted is derived from at least two sources: (1) the dehalogenation of 5-bromouracil derived from BCDR, and (2) the inhibition of the enzymic degradation of uracil (derived from normal metabolic precursors) in a manner comparable to that reported previously with 6-azathymine.[17, 19] The specific activity of the uracil excreted in the urine was 0·5 per cent of that of the ^{3}H-BCDR administered; in a previous experiment with ^{3}H-IUDR,[4] the specific activity of the uracil excreted in the urine was 3 per cent of that of the IUDR.

Studies in vitro. Findings related to the stability of BCDR to metabolic degradative attack *in vitro* are shown in Table 1. When BCDR was incubated with homogenates of adult mouse liver, as much as 87 per cent of the substrate was recovered intact after incubation for 1 hr; with slices of mouse

TABLE 1. INCUBATION OF BCDR WITH LIVER, BLOOD AND INTESTINAL MUCOSAL PREPARATIONS

Incubation time	BCDR recovered			
	Liver		Whole blood	Dialysed rabbit intestinal mucosa
min	Homogenate %	Slices %	%	%
3	93	—	—	—
10	95	—	—	—
60	87	94	—	—
120	—	—	—	97
180	—	75	—	—
480	—	—	97	—

The incubation medium with liver homogenate (final volume, 3·0 ml) consisted of: 100 μmoles of potassium phosphate buffer (pH 7·4), 120 μmoles of nicotinamide, 200 μmoles of KCl, 50 mg of liver homogenized in 0·25 M sucrose–0·002 *M* $CaCl_2$ and 3·2 μmoles of BCDR; with liver slices (final volume, 3·1 ml): 3·0 ml of Krebs III-phosphate buffer,[20] 70–80 mg of liver slices and 3·2 μmoles of BCDR; with blood: 3 ml of whole human blood with heparin and 4·8 μmoles of BCDR in 0·5 ml of water, gas phase was O_2:CO_2 (95:5); with intestinal mucosal enzyme (final volume, 3·0 ml): 1·5 ml of Krebs III-phosphate buffer, 100 μmoles of ATP, 4·8 μmoles of BCDR and dialysed enzyme preparation (scraped mucosal tissue, freeze-thawed four times, dialysed against water overnight, and the dialysand taken up in Krebs III-phosphate buffer). All incubations were performed at 37° with air as the gas phase, unless indicated otherwise. The incubations were terminated by the addition of perchloric acid (final concentration, 0·2 N) or TCA (final concentration, 5%) and the acid-soluble compounds were separated on Dowex-1 formate columns (1 × 25 cm) by gradient elution with formic acid.

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liver, 94 per cent and 75 per cent of the added BCDR was recovered unchanged after incubation for 1 hr and 3 hr, respectively; 5-bromocytosine was not produced under these conditions. Of BCDR incubated for 6 hr with whole human blood, almost all was recovered unchanged. When BCDR was incubated with a dialysed preparation of either mouse or rabbit intestinal mucosa, rich in enzymic activity for the phosphorolysis of IUDR and BUDR, 97 per cent recovery of the substrate occurred.

DISCUSSION

On the basis of presently available information, the resistance of BCDR to catabolic influences within the intact mouse, as compared to that observed with either BUDR or IUDR, has been disappointing. Nevertheless, quantitative measurements of the sequence and products of biochemical transformations, responsible for this metabolic degradation, should be made with various tissues, including neoplasms, in order to determine whether intrinsic differences exist between BCDR and BUDR which could permit preferential utilization of the former by cells. Such studies, now in progress and reported on in part in the following communication, are concerned with the entrance of BUDR and BCDR into the acid-soluble fraction of cells, the incorporation of these compounds into DNA, and their effects on the threshold of cells to radiation injury.

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Preparation of 5-iodo-2′-deoxycytidine*

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RECENT studies on the biological activities of 5-iodo-2′-deoxyuridine[1–4] and 5-bromo-2′-deoxycytidine[5, 6] have indicated that it should be advantageous to have 5-iodo-2′-deoxycytidine available for comparable studies; however, iodination of 2′-deoxycytidine does not occur as readily as in the case of bromination. Iodination in dilute nitric acid, under similar conditions to those used in the preparation of 5-iodo-2′-deoxyuridine,[1] produced only 5-iodo-cytosine. The standard basic iodination procedure (in the presence of potassium hydroxide and potassium iodide), similar to that used by Johnson and Johns[7] for the iodination of cytosine, led to the production from 2′-deoxycytidine of deaminated materials.

It has been found that 2′-deoxycytidine can be conveniently iodinated in the presence of iodic acid using a method resembling that of Wirth *et al.*[8] for the iodination of aromatic compounds.† The preparation of several batches of the new compound has indicated that solvent, temperature, and the concentration of reactants influence the yield of 5-iodo-2′-deoxycytidine; as a solvent, glacial acetic acid gives a better yield than does water. A considerable amount of 2′-deoxycytidine is converted to a by-product that contains iodine, but does not absorb in the ultraviolet region; the elementary analysis of this substance suggests the formula, $C_9H_{12}O_5N_2I_2$ (possibly 5:6-di-iodo-5:6-di-hydro-2′-deoxyuridine); in addition, a small amount of 5-iodo-cytosine is formed.

In the preparation, on a micromole scale, of 5-iodo-2′-deoxycytidine-^{3}H from tritiated 2′-deoxycytidine, the radioactive compound was purified by the use of a Dowex-1 formate column. Prompt elution of the column was necessary in order to minimize cleavage of the deoxyribonucleoside.

EXPERIMENTAL‡

5-Iodo-2′-deoxycytidine. A mixture of 2′-deoxycytidine hydrochloride§ (2·6 g, 0·01 mole), glacial acetic acid (8 ml), iodic acid (0·9 g), iodine (1·5 g), carbon tetrachloride (2 ml) and water (3 ml) was stirred at 40 °C for 90 min; at this time the iodination was essentially complete (on the basis of ultraviolet absorption, iodination continued to about 80 per cent of the theoretical value). To the now clear solution, water (30 ml) was added and the aqueous solution (after filtering off any solids (0·48 g; by-product) present) was extracted several times with carbon tetrachloride (400 ml). The aqueous phase, after concentration *in vacuo* at 40 °C to dryness, was dissolved in methanol (40 ml) and again concentrated *in vacuo* at 40 °C to dryness. This operation was repeated 5 times in order to remove most of the acetic acid. The yellow crystalline residue obtained was taken up in water (30 ml) and the solution was adjusted to pH 10 with sodium hydroxide (10 N); 5-iodo-2′-deoxycytidine began to precipitate out. Refrigeration of the mixture produced the crude product (1·94 g). After one recrystallization from water, the compound (1·69 g) melted with decomposition at 175–178 °C; λ_{max}^{pH2} 308–309 mμ (ξ 8,150); $\lambda_{max}^{H_2O}$ 293 mμ (ξ 6,020); λ_{max}^{pH11} 294 mμ (ξ 5,930); $\lambda_{max}^{95\%\ EtOH}$ 294 mμ (ξ 5,750). *Analysis*—calcd. for $C_9H_{12}O_4N_2I$: C, 30·61; H, 3·43; N, 11·90; I, 35·94; found: C, 30·68; H, 3·52; N, 11·95; I, 35·82. The mother liquor and all the recrystallization filtrates were combined and the solution was adjusted to pH 10 with sodium hydroxide (10 N). The solution was then put on a column of charcoal‖, 2·5 × 12 cm; this was washed quickly with distilled water until free of halides (4–6 l.). The product was eluted from the column with 95% ethanol (5–7 l.) and the solvent was removed *in vacuo* at 40 °C. The residue crystallized to give additional 5-iodo-2′-deoxycytidine (0·5 g), bringing the over-all yield to about 62 per cent.

The solid by-product was dissolved in tetrahydrofuran and reprecipitated by addition of water, m.p. 128–132 °C (decomposition); analysis–calcd. for $C_9H_{12}O_5N_2I_2$: C, 22·42; H, 2·50; N, 5·81; I, 52·65; found: C, 22·69; H, 2·37; N, 5·37; I, 52·21.

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‡ The melting points are uncorrected. Analyses by Schwartzkopf Microanalytical Labs, Woodside, N.Y., and by Midwest Microlab, Inc., Indianapolis, Ind.

§ Purchased from California Corp. for Biochemical Research, Los Angeles, Cal.

‖ Composed of activated carbon, grade 20 × 40, Atlas Powder Co., Wilmington, Delaware.

5-Iodo-2′-deoxycytidine-^{3}*H.* A solution of tritiated 2′-deoxycytidine* in 50 per cent ethanol (2 ml, 2 mc, 1·82 μmole) was reduced to dryness at 40° and vacuum-dried at room temperature. To the dried residue were added non-radioactive 2′-deoxycytidine hydrochloride (2·23 mg, 8·46 μmoles) in sufficient amount to bring the specific activity of the final mixture to 2 mc/10.28 μmoles (194 μc/μmole), dilute hydrochloric acid (0·1 ml, 0·02 N), a solution of iodic acid in water (0·06 ml, 45 mg/ml), and a solution of iodine in carbon tetrachloride (0·1 ml, 20 mg/ml). The mixture was agitated at 40° for 3 hr and taken up in water (1 ml). The carbon tetrachloride layer was separated and washed with a little water (0·2 ml). The aqueous layers were combined and adjusted to about pH 11 with sodium hydroxide (1 N), divided into two equal portions, and put on two 30 × 1-cm Dowex-1 formate columns. These were eluted with water (1 l.) which was agitated magnetically and into which was introduced dropwise, in a continuous manner, 0·025 N formic acid. The eluate was collected in 1-ml fractions; yield, 1366 μg, specific activity, 76 μc/μmole.

* Purchased from Schwarz Bio-Research, Inc., Mt. Vernon, New York.

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Studies on the GABA pathway–II.
The lack of effect of pyridoxal phosphate on GABA-KGA transaminase inhibition induced by amino-oxyacetic acid

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IT WAS reported previously[1] that amino-oxyacetic acid (AOAA) is an effective inhibitor of γ-aminobutyric acid (GABA, α-ketoglutaric acid (KGA) transaminase in the brain in five species of animals, inducing elevated levels of γ-aminobutyric acid. Further, it was reported that with a GABA–KGA transaminase preparation of *E. coli*, the kinetics of inhibition indicated that AOAA is a strictly competitive inhibitor for both substrates of the enzyme. Since the brain enzyme is also inhibited by AOAA, it was assumed that this agent was also competitively inhibiting this enzyme in a similar manner.

In animals it was found that toxic doses of AOAA cause convulsions which terminate in a respiratory death.[1] These convulsions can be prevented by the administration of a variety of different compounds which have in common an aldehydic or ketonic group.[2] One such compound of the series studied was pyridoxal phosphate. In common with other transaminases, GABA–KGA transaminase requires pyridoxal phosphate,[3] so that the question arose whether high concentrations of pyridoxal

phosphate might be capable of reversing the inhibition of GABA–KGA transaminase induced by AOAA.

As observed by Wallach, AOAA at a concentration of $3{\cdot}3 \times 10^{-2}$M has no effect on the succinic semialdehyde dehydrogenase from *E. coli* ATCC-26.[1] Thus, the coupled system of GABA–KGA transaminase and succinic semialdehyde dehydrogenase from this organism was a valid test system for these studies, and was composed in the following manner. To a Beckman DU Cuvette was added 600 μmoles of Tris buffer pH 8·35, 5 μmoles of 2-mercaptoethanol, 6 μmoles of KGA, 1 mg of TPN and 250–300 units of the enzyme preparation.* Additions of AOAA and pyridoxal phosphate were made in the concentrations to be indicated. In all cases, the final volume in each cell was 3 ml. After addition of the indicated components, the contents of the cell were well mixed by inversion, the reaction was started by addition of GABA (with an immediate second mixing of the cell contents), and an initial reading at 340 mμ was taken. Readings were made at 1-min intervals for 5 min. The initial reading was then subtracted from the reading at 5 min in order to determine the extent of TPNH formation.

As a control, the system was run at three concentrations of GABA, as shown in Fig. 1, Curve A. When AOAA was added at a final concentration of $6{\cdot}6 \times 10^{-6}$M, a 50 per cent inhibition of the reac-

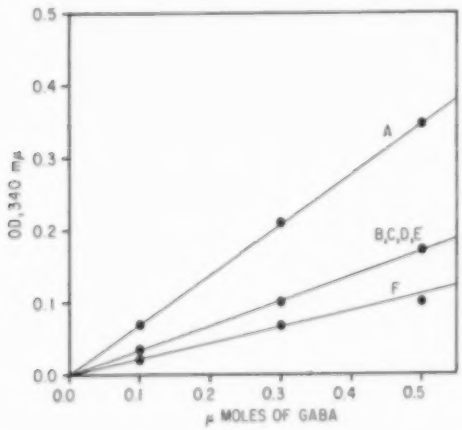

FIG. 1. The contents of each Beckman cuvette were as follows: 600 μmoles of Tris buffer, pH 8·35, 5 μmoles of 2-mercaptoethanol, 6 μmoles of KGA, 1 mg of TPN, 250–300 units of the coupled enzyme system from *E. coli*, and water to a final volume of 3 ml. Additions of AOAA and pyridoxal phosphate and GABA were as indicated in the text. The reaction was run at room temperature.

tion rate was observed, as shown in Fig. 1, Curve B; pyridoxal phosphate was then added to the cuvettes to produce final concentrations of $6{\cdot}6 \times 10^{-6}$M, $6{\cdot}6 \times 10^{-5}$M and $6{\cdot}6 \times 10^{-4}$M. Thus, there was present a 1-, 10- or 100-fold higher concentration of pyridoxal phosphate, as compared to the fixed concentration of inhibitor. The curves shown in Fig. 1, C, D, and E, were identical with Curve B, a circumstance which shows that at these concentrations there is no reversal by the cofactor of the AOAA-induced, inhibition of the enzyme. When the concentration of pyridoxal phosphate was increased to $6{\cdot}6 \times 10^{-3}$M, the enzyme was markedly inhibited, as shown in Curve F of Fig. 1. This concentration of the cofactor had no effect on succinic semialdehyde dehydrogenase. [This inhibitory effect of high concentrations of pyridoxal phosphate on GABA-KGA transaminase has also been

* A unit of the coupled enzyme preparation is defined as that amount of enzyme which will induce an OD change of 0·001 per min under the specified conditions of assay above and with the addition of 6 μmoles of GABA.

observed by C. F. Baxter (Private communication)]. Thus, no concentration of pyridoxal phosphate in the range employed reversed the inhibition induced by AOAA. These results are fully in agreement with the previously reported observation that the mechanism of the inhibition by AOAA of GABA–KGA transaminase from *E. coli* was of the strictly competitive type for both substrates of the enzyme. It was also definitive proof that under the conditions of the assay there was no significant degree of complexing between AOAA and pyridoxal phosphate, since a decreasing degree of inhibition would have been expected with increasing concentrations of the cofactor.

While it was most unlikely that AOAA, an inhibitor of both the bacterial and mammalian enzymes, could inhibit the two enzymes by different mechanisms, this point was checked, as is shown by the experiment of Table 1. It is evident not only that pyridoxal phosphate does not reverse the inhibition,

TABLE 1. THE NONREVERSAL OF GABA–KGA TRANSAMINASE DERIVED FROM BRAIN BY PYRIDOXAL PHOSPHATE (PY. PHOS.)

Tube contents	Complete system*	Complete system + 1×10^{-4}M (Py. Phos.)	Complete system + 1×10^{-3}M (Py. Phos.)	Complete system + 1×10^{-5}M U-7524	Complete system + 1×10^{-4}M (Py. Phos.) 1×10^{-5}M U-7524	Complete system + 1×10^{-3}M (Py. Phos.) 1×10^{-5}M U-7524
Concentration of Py. Phos. over U-7524	—	—	—	1 x	10 x	100 x
Percentage synthesis	100	110†	94‡	8	0	0
Reversal	—	—	—	—	0	0

* Complete system contains:
40 μmoles GABA
40 μmoles KGA
0·15 μmoles Py. Phos. (needed to supplement brain enzyme)
100 μmoles Borate pH 8·2
Enzyme from cat brain

† Note stimulation of system by pyridoxal phosphate at this concentration.

‡ Note depression of synthesis at this concentration of pyridoxal phosphate.

but also that excessive concentrations of the cofactor are of themselves somewhat inhibitory. This same effect has been reported previously for the mammalian transaminase by Baxter and Roberts.[3] The mechanism of the inhibition by pyridoxal phosphate has not been explained.

DISCUSSION

It seems reasonable to suggest that AOAA is a competitive inhibitor of GABA–KGA transaminase in brain. Although the mechanism of the convulsions induced by excessive doses of AOAA remains obscure, the fact that several compounds encompassing an aldehydic or ketonic function do reverse these convulsions points to either one of two possibilities. It is well known that AOAA complexes with carbonyl compounds (including pyridoxal phosphate at higher concentrations than those employed here) and at this time, a simple complex formation between this type of compound and AOAA cannot be ruled out. If the brain levels of one or more critical carbonyl compounds were drastically reduced by complex formation with AOAA, convulsions might well ensue. On the other hand, when excessive doses of AOAA are given to an animal, the GABA–KGA transaminase in the brain could be inhibited completely for a period of several hours. This should result not only in increased concentrations of GABA (and in fact this has been observed), but also a very marked depletion in the brain of the succinic semialdehyde which arises from the transamination of GABA. Since GABA and succinic semialdehyde are the two compounds which are unique to the GABA pathway, it is possible that there may be a reciprocal relationship between these two compounds in brain which has hitherto been overlooked. Accordingly, it is suggested that succinic semialdehyde may play a physiological role and that the convulsions seen with large doses of AOAA may result

from a depletion of succinic semialdehyde. The role of this aldehyde in the brain may be as important as GABA and investigation of its function may prove fruitful.

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5-Bromo-2′-deoxycytidine (BCDR)–II. Studies with murine neoplastic cells in culture and *in vitro*

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IN CONJUNCTION with studies of the stability of 5-bromo-2′-deoxycytidine (BCDR) to metabolic degradative attack *in vivo*,[1] the presently reported work was undertaken to investigate the toxicity, metabolism and mechanism of action of BCDR with mammalian neoplastic cells, both in culture and under conditions of incubation *in vitro*.

Effect of BCDR on murine neoplastic mast cells grown in culture. The culture medium,[2] cell line (murine neoplastic mast cells, P815Y),[3] and techniques[4] have been described previously, with the exception that the Coulter Cell Counter was used for determinations of the number of cells.

BCDR in graded concentrations, 1–400 μM ,in the medium progressively inhibited the growth of the cells, although complete inhibition did not occur even with 400 μM. With that level, cell reproduction was limited to only two divisions; while with the same level of 5-iodo-2′-deoxyuridine (IUDR) or 5-bromo-2′-deoxyuridine (BUDR), growth was limited to but a single division. Following exposure of the cells to BCDR for three cell-generations and subsequent transfer to drug-free medium, a progressive decrease in cell viability occurred with increasing concentration of the agent.

Inhibition of growth of BCDR could be prevented partially by addition to the medium of either thymidine (TDR) or 2′-deoxycytidine (CDR); however, much higher levels of CDR than of TDR were required for comparable degrees of prevention of growth inhibition, a finding which supports the concept that BCDR and TDR are handled analogously by the cells. However, complete reversal by TDR could not be obtained, since levels greater than about 60 μM are toxic to the cells, a phenomenon which has been described previously and explained.[5]

Both BUDR and BCDR partially prevented the inhibition of cell growth caused by otherwise totally inhibitory levels of 5-fluoro-2′-deoxyuridine (FUDR), but neither compound was as effective as TDR.

The uptake and metabolism of ^{3}H-BUDR and ^{3}H-BCDR *in vitro* were compared using suspensions of cells, 5×10^7 per ml, incubated with tracer quantities of nucleosides of high specific activity. After periods of incubation of 5, 15 and 30 min, respectively, the tubes were chilled, carrier BUDR and BCDR were added, and the cells were sedimented by centrifugation. The surface of the packed cells and the walls of the tube were washed once with fresh medium to remove most of the residual compound; the cell-pack was then extracted three times with cold 5% trichloroacetic acid (TCA), followed by extraction with 5% TCA at 90°C for 30 min. After the TCA was removed from the extracts with ether and water was removed *in vacuo*, the residues derived from the extractions with cold TCA were chromatographed on Whatman no. 1 filter paper in butanol:water (344:56, v/v). No significant

radioactivity on the chromatogram was associated with the carrier BUDR derived from the cold acid soluble extracts of the cells incubated with ^{3}H-BCDR. However, these extracts contained a phosphorylated derivative of BUDR which had the same R_f on Whatman no. 1 filter paper, developed in isopropanol:HCl:water (260:64:76, v/v), as that of authentic deoxyuridine 5′-monophosphate; much higher levels of the same phosphorylated derivative were found in the cold acid-soluble extracts of the cells incubated with ^{3}H-BUDR. The hot TCA-soluble extracts of the cold acid-insoluble residues also contained radioativity which resulted from incorporation of the radioactive precursors into the cellular DNA; moreover, much more radioactivity was present in the extracts derived from cells incubated with ^{3}H-BUDR than was present in those derived from cells incubated in the presence of ^{3}H-BCDR. This finding suggests that BCDR was converted to phosphorylated derivatives of BUDR, with subsequent incorporation into the DNA of the cell, and that this occurred at a more rapid rate with BUDR than with BCDR. In comparison with the results obtained with ^{3}H-BUDR, those with ^{3}H-BCDR might be attributed to either reduced transport of ^{3}H-BCDR through the cell wall or to reduced conversion of BCDR to BUDR-phosphates *via* deamination and phosphorylation, or to both circumstances.

Enzymically hydrolysed DNA prepared from cells incubated with ^{3}H-BCDR contained essentially all its radioactivity as 5-bromo-2′-deoxyuridine 5′-monophosphate (Table 1); this fraction replaced an equivalent amount of thymidylic acid (dTMP) (46 per cent of that of the newly synthesized DNA); the sum of these nucleotides, 2·24 μmoles, corresponded almost exactly to the amount of 2′-deoxyadenylic acid (dAMP) isolated, 2·14 μmoles, in accordance with the Watson-Crick[7] formulation for base-pairing.

TABLE 1. INCORPORATION OF BCDR AS 5-BROMO-2′-DEOXYURIDYLIC ACID INTO THE DNA OF MURINE NEOPLASTIC MAST CELLS (P815Y) IN CULTURE

Deoxyribonucleotide	μMoles of nucleotide in DNA	Per cent replacement of thymidylic acid by BUDR-5′-P
dAMP	2·14	—
dTMP	1·40	—
BUDR-5′-P	0·84	46·4

Murine neoplastic mast cells (P815Y) were grown in mass culture for slightly more than two cell divisions in the presence of 2×10^{-5} M ^{3}H-BCDR (1·45 μC/μmole). The washed harvested cells (0·9 g) were homogenzied in 0·25 M sucrose–0·002 M $CaCl_2$ and the nuclei were sedimented by centrifugation at 800 g for 15 min, washed once with fresh sucrose–$CaCl_2$, and the supernatant fractions discarded. The nuclei were dissolved in cold 1·7 M NaCl which was then added to 2 vols of 95% ethanol to precipitate the nucleoprotein. The precipitate was redissolved in 1·7 M NaCl and the solution was homogenized with an equal volume of chloroform:octyl alcohol (5:1) three times, in order to remove the protein. The DNA was again precipitated by the addition of the NaCl solution to ethanol and the dried preparation was degraded enzymically with pancreatic deoxyribonuclease (Worthington), then with a purified snake venom phosphodiesterase which had no significant deaminase activity. The resulting mixture of deoxyribonucleotides was separated on Dowex-1 formate columns (1 × 25 cm) by gradient elution with a solution of ammonium formate (1 M, pH 4·1).[6] The quantity of each deoxyribonucleotide recovered was determined spectrophotometrically on the pooled samples; the concentration of BUDR-5′-P was calculated on the basis of the specific activity of the initial ^{3}H-BCDR and the compound was further characterized as BUDR by paper chromatography in butanol:water, following dephosphorylation of the nucleotide with alkaline phosphatase.

The pathway by which BCDR is converted to phosphorylated derivatives of BUDR in the P815Y cell can be deduced from data concerned with cross-resistance to BCDR (and other related compounds) of a cloned sub-line (P815Y/FUDR) selected for resistance to FUDR.[5] A much higher level (about 1000-fold higher) of FUDR (50 per cent growth inhibitory concentration: $2·6 \times 10^{-6}$ M) was required to inhibit the growth of these cells than was needed for the inhibition of the growth of the parent cells. The P815Y/FUDR cells also were cross-resistant to levels of either TDR, IUDR or BUDR which were profoundly toxic for the sensitive parent strain; however, the cells were *not* resistant to 5-fluoro-2′-deoxycytidine (FCDR). Resistance to the first three cited compounds has been shown to

result from a greatly reduced level of thymidine kinase activity in the P815Y/FUDR cell.[5] Lack of cross-resistance to FCDR implies that the metabolic block which results from deletion or suppression of thymidine kinase activity may be circumvented by direct phosphorylation and deamination of FCDR, with intracellular formation of the 5′-monophosphate of FUDR, the active inhibitor of thymidylic acid synthetase.[8, 9] Cross-resistance to BCDR infers that, in the P815Y/FUDR cells, activation of this compound does *not* proceed to a significant degree by the same pathway as does FCDR. *A priori*, its conversion to phosphorylated derivatives of BUDR in the parent line may occur following deamination. Assay of enzyme preparations of the parent cells for the presence or absence of significant activity for the deamination of BCDR to BUDR, prior to phosphorylation, will be necessary to confirm this hypothesis.

Deaminase activity in vitro *of a cell-free preparation of murine L5178Y lymphoblasts.* Cells were harvested 6 days following the inoculation of AKR × DBA/2 F_1-hybrid mice with L5178Y ascites cells; after centrifugation the cells were washed once with saline. Packed cells (3 ml), Tris buffer (0·05 M, pH 7·9) and glass beads (200 micron, 7 ml) were shaken for 20 sec in a Nossal vibrator. Following filtration through glass wool, the filtrate was centrifuged at 10,000g for 10 min. The reaction mixture (1·2 ml) contained $MgCl_2$ (5·5 μmoles), ATP (110 μmoles), Tris buffer (110 μmoles, pH 7·9), ^{3}H-BCDR (0·1 μmole, 6×10^4 cpm), and cell-free extract (0·5 ml). The reaction was terminated, after incubation for 90 min at 37 °C, by the addition of TCA. Both the composition of the reaction mixture and the conditions used were similar to those described by Weissman *et al.*[10]

The acid-soluble fraction was separated into two radioactive areas by paper chromatography in the ethylacetate:phosphate buffer system.[11] One area, which contained about 9 per cent of the total radioactivity, was identified as BUDR. The other area, which was closely associated with the point of origin, was eluted and rechromatographed in the butanol:ammonia system;[12] essentially all radioactivity of this latter area migrated with authentic BCDR. No radioactivity was observed at the point of origin, in which nucleotides, if formed, would have been present. Under these conditions of incubation, the formation of thymidylic acid from ^{3}H-TDR has been demonstrated previously.[13] Thus, the cell-preparations contained enzymic activity for the deamination of BCDR, but the data do not establish whether this deamination occurred before or after phosphorylation; it is conceivable that the extracts may have rapidly hydrolyzed any phosphorylated derivatives of either BCDR or BUDR, or both.

Effect of BCDR on the utilization of TDR for the biosynthesis of DNA by various neoplasms in vitro. Although studies of neoplastic mast cells in culture (see earlier section) demonstrated the extensive incorporation into DNA of 5-bromo-2′-deoxyuridylic acid, derived from BCDR, another possible site of action of this agent could involve an inhibition, at either nucleoside or nucleotide levels, of the utilization of TDR for the biosynthesis of DNA-thymine. Evidence for this type of inhibition has been provided in earlier studies in which the capacity of either orotic acid or thymidine to serve as a precursor of DNA-thymine was markedly reduced by BUDR and IUDR[14, 15]; of these compounds, BUDR was the more active. The decreased incorporation of TDR in the presence of IUDR reflects an inhibition of the itilization of either TDR, thymidylic acid or the triphosphate of TDR, presumably by IUDR or the corresponding phosphorylated derivative.[13]

Since BCDR also inhibits the incorporation of ^{3}H-TDR into the DNA-thymine of intact Ehrlich ascites carcinoma cells and L5178Y lymphoblasts (Table 2), the question arises as to whether BCDR or its phosphorylated derivatives possess antimetabolic activity *prior* to deamination. Evidence for deaminase activity in cell-free extracts of the L5178Y cells has been provided; accordingly, the extent of inhibition observed in Table 2 could be accounted for by a deamination of the added BCDR to the extent of only 10–20 per cent.

It appears that BUDR is more potent than BCDR as an inhibitor of the utilization of TDR for the formation of DNA-thymine. Thus, BUDR, at a ratio of 20:1, inhibited by 96 per cent the utilization of TDR for the biosynthesis of DNA-thymine by murine Ehrlich ascites carcinoma cells *in vitro*,[14] whereas BCDR, even at a ratio of 50:1, exerted only 53 per cent inhibition (Table 2).

DISCUSSION

The results indicate that with three types of murine neoplastic cells (mast cells, P815Y; lymphoblasts, L5178Y; and Ehrlich ascites carcinoma cells), BCDR is deaminated; incorporation into DNA, in the form of BUDR-5′-P in place of thymidylic acid, has been demonstrated with the mast cells.

Although not unequivocally established that BCDR can be deaminated *directly* (i.e., without prior phosphorylation), the studies with mast cells resistant to BCDR (but not to FCDR), strongly indicate that primary deamination of BCDR does occur with these cells. In other cases, however, the formation of BCDR-5′-P, with deamination by deoxycytidylate deaminase, to form BUDR-5′-P, also may occur. Thus, a possibility remains that this latter pathway could predominate in some types

TABLE 2. EFFECT OF BCDR ON THE UTILIZATION OF ^{3}H-THYMIDINE FOR THE BIOSYNTHESIS OF DNA-THYMINE BY TWO MURINE NEOPLASMS *in vitro**

Cells	Amount of BCDR in μmoles	Relative specific activity of isolated DNA-thymine†
Ehrlich ascites Carcinoma cells	None	1·00
	0·5	0·66
	5·0	0·47
L5178Y Lymphoblasts	None	1·00
	0·5	1·06
	5·0	0·65

* The reaction mixtures (final volume, 2·7 ml) contained packed cells (0·25 ml), horse serum (0·2 ml), ^{3}H-TDR (0·1 μmole, 5 μc), in the absence or presence of BCDR in the amounts listed in the table, and Totter's modification of Chamber's solution.[16] The incubations were conducted in duplicate in 20-ml beakers in a Dubnoff metabolic shaker at 37° C (air); agitation was at 90 cycles per min for 4 hr.

† The specific activity of the control DNA-thymine was equated to 1·00.

of neoplastic cells, and this is deserving of further investigation, despite the finding that, in mice,[1] BCDR is less resistant to metabolic degradation than various studies *in vitro* had suggetsed. Since 5-bromocytosine does not appear to be formed from BCDR, at least in mouse liver *in vitro*,[1] dehalogenation to free uracil presumably reflects the intermediation of 5-bromouracil derived from deaminated BCDR, despite earlier indications that BCDR did not participate in reactions leading to the removal of the deoxyribose-moiety.[17] Accordingly, the problems for the immediate future involve direct determinations of the relative rates and mechanisms of anabolism and catabolism of BUDR and BCDR, particularly in man. Possibly of even greater potential chemotherapeutic interest will be comparable studies with IUDR and 5-iodo-2′- deoxycytidine.[18]

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Influence of nicotinic acid and nicotinamide on diphosphopyridine nucleotide (DPN) synthesis in ascites tumor cells*

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IN THE biosynthesis of DPN from nicotinic acid (NA) and nicotinamide (NAM), desamido-DPN was identified as the common precursor in human erythrocytes and mouse liver tissue.[1, 2] Comparing the relative abilities of both types of cells to synthesize DPN from the respective vitamins, however, it is apparent that erythrocytes depend almost totally on NA and cannot utilize NAM to any appreciable extent, while liver tissue shows a preference for NAM as a DPN-precursor.

Roitt[3] demonstrated that alkylating agents of the ethyleneimine type inhibit glycolysis in tumor cells by interference with the synthesis of DPN. The decrease in the intra-cellular concentration of DPN associated with the action of the alkylating agent could be prevented when NAM was added to the incubation medium;[4] NA was ineffective in this respect. Concluding from these experiments that tumor cells may depend on NAM rather than NA for DPN-synthesis, it was desirable to investigate the specific utilization of these vitamins by a tumor and to compare the results with the biochemical transformations observed in a non-neoplastic tissue of the same animal.

From a group of 12 white Swiss–Webster mice (weight 50–60 g) with Ehrlich ascites tumor, 4 animals were injected intramuscularly with NA, 170 mg/kg, 4 received NAM in equal doses, and the remainder served as controls. Ten hours after the injections, concentrations of oxidized DPN in liver and tumor homogenates were estimated spectrophotometrically, using crystalline yeast alcohol dehydrogenase according to the method of Holzer.[5]

The results (see Table 1) indicate that the capacity of the tumor cells for DPN-synthesis from either NA or NAM is only about one-tenth that of liver tissue of the same animal. Comparing the specific utilization of the precursors for the synthesis of DPN, NAM was approximately four times as effective as NA in both liver and tumor, suggesting a similar biochemical mechanism for DPN-synthesis in these tissues. The limited response of the tumor cell is not related to insufficient transfer of the precursors into the ascitic fluid. No significant different in DPN levels was observed when NAM and NA were injected intraperitoneally instead of intramuscularly (unpublished data). The possibility that the tumor cell has a poor capacity to incorporate the precursors can be disregarded, since Holzer and Boltze[6] using ^{14}C-labeled NAM and NA showed that both vitamins penetrate freely into ascites tumor cells. Further investigations are needed to determine whether the same intermediates and enzymic sequences occur in this tumor.

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TABLE I. DPN-CONCENTRATIONS IN ASCITES TUMOR CELLS AND LIVER TISSUE OF MICE 10 HR AFTER INTRAMUSCULAR ADMINISTRATION OF NICOTINAMIDE AND NICOTINIC ACID (170 MG/KG)

	No. of animal	Ascites tumor cells (μg of DPN/ml of packed cells)	Liver tissue (μg of DPN/g of wet weight)
A. Control animals	1	208	193
	2	194	263
	3	194	232
	4	197	215
		Mean 198	225
		S.D. ±7	±30
B. Nicotinamide	5	317	1451
	6	—	1351
	7	285	1260
	8	290	1520
		Mean 297 (+50%)	1395 (+521%)
		S.D. ±17	±114
C. Nicotinic acid	9	227	430
	10	230	530
	11	—	550
	12	235	655
		Mean 230 (+16%)	541 (+141%)
		S.D. ±4	±92

Two samples were lost by overalkalization. *t*-Test comparisons are significant at or beyond the 0·01 level of probability for B against A, C against A, and B against C, with respect to both ascites tumor cells and liver tissue. Figures in parentheses represent average increases over the control values.

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Separation of thymidine and its mono-, di- and tri-phosphate by paper chromatography

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THYMIDINE (TDR) and its various 5′-phosphorylated derivatives (thymidylic acid (TMP), thymidine diphosphate (TDP), and thymidine triphosphate (TTP)) have been separated readily from each other by column chromatography;[1, 2] however, it has been difficult to obtain clean separations using paper chromatography.[3] Although TDR and TMP were easily separable on paper, TDP and TTP, under the same conditions, appreciably overlapped, and the results could be interpreted only in a semi-quantitative manner.[3] Since paper chromatography often permits the separation of micro-quantities of materials, and is very convenient when dealing with a large number of samples, such a method for the efficient separation of TDR, TMP, TDP and TTP was sought. The present report describes a method, using Whatman cellulose paper AE30, which effectively separates the four compounds.

The paper strip to be used in the separation was washed successively with formic acid (0·1 N), ammonium formate (0·5 N) and water for 24 hr. After the addition, at the top of the dried paper strip, of the solution containing the thymine-derivatives, and re-drying, the first solvent, formic acid (0·01 N), was used to separate TDR from the other compounds. In this solvent system, TDR moved with the solvent front, which was permitted to travel almost to the end of the paper. The paper was re-dried before elution with the second solvent system, ammonium formate (0·06 N, pH 5·0), which was allowed to flow for about two-thirds of the distance traversed by the first solvent system. Such a

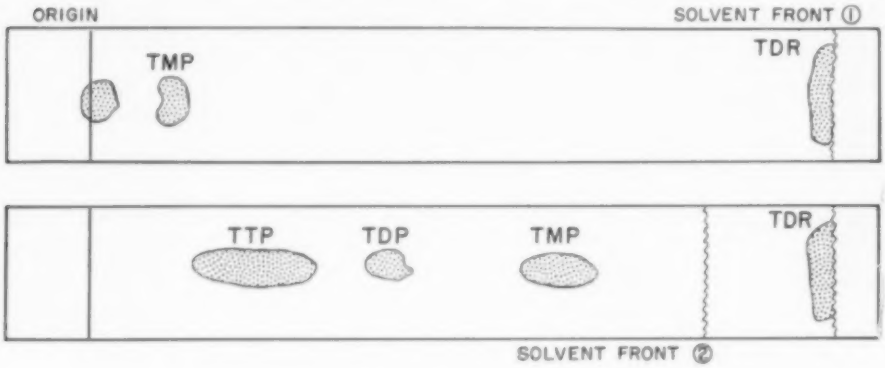

FIG. 1 Paper chromatograms

separation is desirable particularly when studying the metabolism of radioactive thymidine. The various phosphorylated derivatives of TDR, shown in the Fig 1, were located with the aid of an ultraviolet lamp; each component could be eluted with dilute hydrochloric acid (0·1 N) with essentially 100 per cent recovery. The minimum quantity of each compound which could be discerned clearly was 0·2 μmole, although smaller quantities were detectable.

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Inhibition of crotonyl coenzyme A-reducing activity of liver microsomes by 1:1:3-tricyano-2-amino-1-propene (U-9189)

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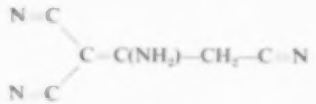
THE decreased capacity of diabetic rat liver to synthesize fatty acids has been related, in part, to the decreased activity of crotonyl coenzyme A (crot-CoA)-reducing enzymes in particle fractions from the liver [1] We have found that the activity of this enzymic reaction in rabbit and rat liver microsomes

is inhibited by 1:1:3-tricyano-2-amino-1-propene (U-9189),[2] a compound also shown[3] to be an inhibitor of oxidative phosphorylation.



(U-9189)

The standard assay mixture consisted of 600 μmoles of Tris buffer, pH 7·5, 60 μmoles of $MgCl_2$, 0·20–0·25 μmoles of reduced triphosphopyridine nucleotide* (TPNH), a suspension of washed liver microsomes (collected in 0·25 M sucrose) in 0·1 M phosphate buffer, pH 7·5, with 0·5% sodium cholate, and water to 1·8 ml. After measuring any endogenous TPNH utilization, 0·2 ml (0·05 μmoles) of crot-CoA solution (prepared according to Goldman[4]) was added, and the decrease in absorbance at 340 mμ (compared to a mixture containing all ingredients except TPNH) was measured at 30-sec intervals for 5–10 min in the Beckman DU-spectrophotometer. All reagents were held at 37°, and the cuvette compartment also was controlled at this temperature.

A rabbit liver microsomal preparation which utilized TPNH at a net of 1·3 mμmoles per min per mg protein was completely inhibited when U-9189 was present at 5×10^{-4} M. The inhibitions were noted when U-9189 was added either before substrate addition or after a 5-min measurement of activity in the complete system. At 5×10^{-5} M, U-9189 inhibited the system by about 10–15 per cent. If sufficient microsal suspension was present to produce a signifiant rate of TPNH utilization before substrate addition, U-9189, 5×10^{-4} M was without effect on this endogenous consumption.

Table 1 presents data from two preparations of rat liver microsomes in the presence of varying concentrations of U-9189 added prior to substrate. In another experiment, a concentration of $4·5 \times$

TABLE 1. EFFECT OF U-9189 ON CROTONYL CoA-REDUCING ACTIVITY IN RAT LIVER MICROSOMES

Final molar conc. U-9189	Experiment 1		Experiment 2	
	mμmoles TPNH utilized per min per mg protein	Per cent inhibition	mμmoles TPNH utilized per min per mg protein	Per cent inhibition
0	2·1		2·4	
5×10^{-4}	0·25	90		
4×10^{-4}			0	100
$2·5 \times 10^{-4}$	1·0	52	0·75	70
1×10^{-4}	1·4	35	1·8	25
$7·5 \times 10^{-5}$	1·5	30		
5×10^{-5}	1·6	25		

The assay system is described in the text. For a particular microsomal suspension, maximum variation of duplicate measurements of the utilization of TPNH was ±5 per cent.

10^{-4} M, following a 5-min measurement of activity in the complete system, resulted in complete cessation of TPNH utilization, while a concentration of 1×10^{-5} M reduced the rate by about 10–15 per cent.

Final concentrations of 10^{-3} M KCN, acetonitrile or malononitrile had no inhibitory effect, nor did two compounds† related to U-9189, $C_2H_5—O—\underset{\underset{O}{\|}}{C}—\underset{\underset{CN}{|}}{C}—\underset{\underset{NH_2}{|}}{C}—CH_3$ (U-14, 508), 4×10^{-4} M final concentration, and $C_2H_5—O—\underset{\underset{O}{\|}}{C}—\underset{\underset{CN}{|}}{C}=\underset{\underset{NH_2}{|}}{C}—\underset{\underset{O}{\|}}{C}—O—C_2H_5$ (U-13, 758), 9×10^{-4} M.

* Sigma Chemical Co. Solutions were prepared just before use and concentrations determined spectrophotometrically.

† Prepared by Dr. G. A. Youngdale.

In contrast to the effect on the utilization of TPNH in crot-CoA-reduction, U-9189 had little or on effect on glutathione reductase,* another system in which the reduced nucleotide is required. Concentrations between 5×10^{-4} M and 2×10^{-3} M exhibited variable and inconsistent inhibitions of from 10 to 25 per cent. The order of introduction of U-9189 was of no significance.

Thus, 1:1:3-tricyano-2-amino-1-propene not only is an inhibitor of an enzymic step important in the synthesis of fatty acids in liver, but also it may possess a specificity of activity which could facilitate elucidation of the mechanistic details of the reactions involved. Black and Hudson[5] have presented evidence for a flavin prosthetic group in yeast glutathione reductase, and Lynen recently reported[6] that flavin mononucleotide is an intermediate in the reduction by TPNH of α, β-unsaturated acyl CoA-compounds during fatty acid synthesis by yeast. Wakil has suggested[7] that precedence favours the flavin intermediate in TPNH-double bond reductions. If this generalization is valid, in the microsomal system it seems most probable that U-9189 is actually interfering with hydrogen-transfer from a flavin to the model substrate.

* A preparation from yeast obtained from Sigma Chemical Co.

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SRS-S concentrates from normal swine lung without anaphylaxis

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THE release of a smooth muscle-stimulating substance, in addition to histamine, from the lung tissue of guinea pig during anaphylaxis has been reported,[1-5] and is of interest in research in allergy. This "substance" has not been proven by organic chemical data to be a single substance, but is characterized primarily by biological effects and the relation of biological response to chemical fractionation. This substance causes a prolonged contraction of isolated smooth muscle, in contrast to the rapid contraction caused by histamine; consequently, it has been designated "slow-reacting substance" or SRS[1, 2] or SRS-A[5] (A for anaphylaxis).

Previous methods[1-5] for extraction of SRS or SRS-A from guinea pigs required sensitization of the animals with antigen, usually egg albumin. The lungs were then removed, and anaphylaxis was induced by perfusion or incubation of the sensitized lungs with antigen solution. The aqueous effluents contained SRS and histamine only after anaphylaxis. The existence of SRS in these effluents was based on observations of the characteristic contraction of the guinea pig ileum after suppression of the action of histamine with antihistaminic agents.[2] In our study, this procedure served as the basis of an assay[5, 6] for activity of samples.

The preparation of concentrates of SRS for biological and chemical studies is greatly hampered by the small quantities available from the lungs of guinea pigs and by the sensitization requirement. Although the background research seemed to preclude the presence of SRS in normal lung tissue, lungs from swine have been investigated as a source for SRS, and very active concentrates have been prepared. In the absence of definitive characterizing data on SRS obtained from different sources and

by isolation steps which do and do not include anaphylaxis, we are tentatively designating our active material SRS-S (S for swine).

It was found that Tyrode solution extracted much activity from fresh swine lungs that are relatively free of blood. Background information for our isolation procedure included work by Chakravarty[3] and Brocklehurst.[5, 7] For example, advantage was taken of the fatty acid-like properties of SRS; i.e., water-soluble in weakly basic solution and organic solvent-soluble in acidic solution.

Tissue residue was removed by centrifugation from the Tyrode extract, and salts and proteins were removed by precipitation with alcohol. The alcohol was removed *in vacuo*, and the aqueous solution was acidified. SRS-S was then separated from histamine and other water-soluble components by extraction with chloroform. The chloroform-soluble lipids were fractionated by extraction with sodium bicarbonate solution. The aqueous extract was acidified and extracted with chloroform. Evaporation of the solvent yielded about 50 mg of a histamine-free lipid concentrate from 1 kg of wet tissue; this material contracted the guinea-pig ileum at a level of 0·025–0·125 μg/ml of bathing fluid.

Certain other smooth muscle-stimulating substances have been isolated from blood. In particular, G-acid[8] a fatty acid, has properties similar to SRS. The possibility that SRS-S was derived from residual blood in the lung tissue seems to be unlikely; no activity of SRS-S was found when swine blood was similarly processed.

A basic physiological significance for "slow-reacting substance(s)" acting in possible relationship with histamine in allergic syndromes is not fully elucidated, and could hardly be expected at this time when active concentrates have been available only on a micro scale. The new findings reported herein may facilitate the isolation of a pure substance(s), the elucidation of structure, and comparisons with other substances active in the rather nonspecific assay.

Acknowledgement—In May, 1960, we discussed with Dr. Brocklehurst the possibility of obtaining SRS from lung tissue without sensitization and anaphylaxis; he has since written that during incubation of fragments of swine lung, SRS activity is freed into the bathing fluid. Professor Börje Uvnas has kindly tested a sample of SRS-S and found it to be active.[10] We are grateful to Dr. Brocklehurst for exchange of assay samples and particularly for helpful discussions in his laboratory.

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ANNOUNCEMENT

THE editors of BIOCHEMICAL PHARMACOLOGY feel that a useful purpose would be served by publishing short critical reviews of recent meetings and symposia on subjects of interest to readers of the Journal. These reports should be prepared by someone who has attended the meeting, and should be submitted immediately after the meeting is finished so that they can appear within three months of the actual time of the meeting. The Publishers are prepared to pay a small fee for the preparation of such a report. The Editors would appreciate it if their attention were drawn to suitable meetings and symposia, and if suggestions were made as to who might be qualified and prepared to make such a report. In the first instance all communications should be addressed to the relevant Regional Editor.

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